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(71) Applicant *(for all designated States except US)*: WASHINGTON UNIVERSITY [US/US]; 600 South Euclid Avenue, St. Louis, MO 63110 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: RICHARDS, Eric, J. [US/US]; 4446 Westminster Place, St. Louis, MO 63108 (US). JEDDELOH, Jeffrey, A. [US/US]; 6756 W. Lakeridge Drive, New Market, MD 21774 (US).

(74) Agents: REED, Janet, E. et al.; Dann, Dorfman, Herrell and Skillman, Suite 720, 1601 Market Street, Philadelphia, PA 19103 (US).

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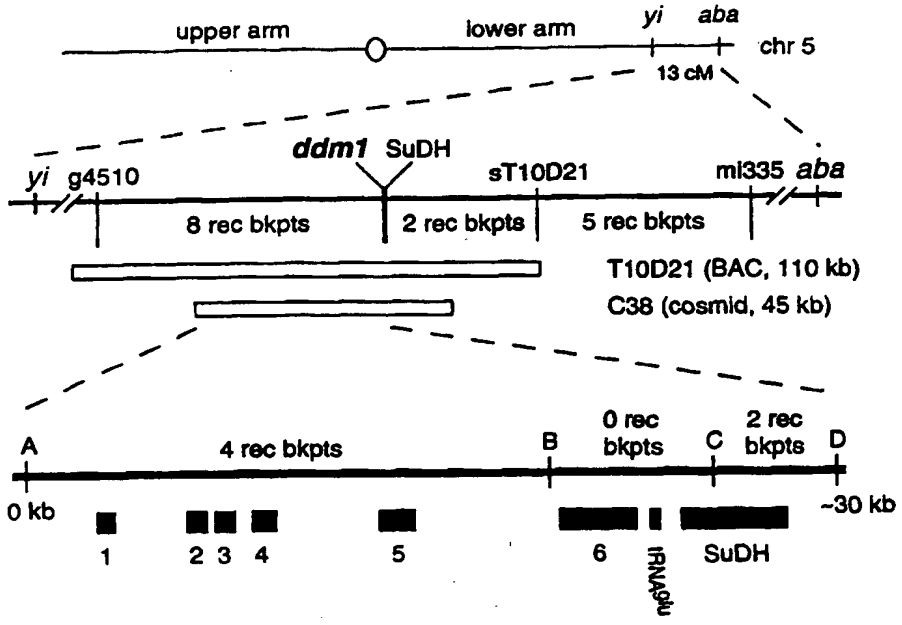


(57) Abstract

A novel gene, *DDM1*, and its encoded protein are provided. The gene was isolated from a region of *Arabidopsis thaliana* chromosome 5. *DDM1* appears to be part of the SWI2/SNF2 family of chromatin–remodeling proteins. Disruption of the gene results in DNA hypomethylation, among other phenotypes. The *DDM1* gene defines a novel member of the DNA methylation system. Methods of using *DDM1*, and transgenic organisms comprising *DDM1*, are also provided.

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# PLANT GENE THAT REGULATES DNA METHYLATION

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the National Science Foundation, Grant Nos. MCB9306266 and BIR9256779.

This application claims priority to U.S. Provisional Application Serial No. 60/_____, filed April 30, 1998, and to U.S. Application No. 09/104,070, filed June 24, 1998 the entireties of which are incorporated by reference herein.

## FIELD OF THE INVENTION

This invention relates to the field of plant molecular biology, genetic engineering and regulation of gene expression. In particular, this invention provides a novel gene, *DDM1*, which plays an important role in the regulation of DNA methylation, and resultant regulation of gene expression, in plant genomic DNA.

## BACKGROUND OF THE INVENTION

Various publications or patents are cited in this application to describe the state of the art to which the invention pertains. Each of these publications or patents is incorporated by reference herein.

Plant genomes contain substantial amounts of 5-methylcytosine. Up to 20-30% of the cytosines are methylated in the nuclear genome of many flowering plants. As in other organisms, methylation of cytosine

residues in plants occurs post-replicatively through the action of cytosine-DNA methyltransferases. Plant DNA methyltransferases have been characterized biochemically, and plant genes encoding these enzymes have been isolated by virtue of their similarity to their mammalian counterparts.

Investigations of native plant genes and transgenic plants containing foreign genes have found a general correlation between transcriptional inactivity and increased DNA methylation, consistent with evidence from mammalian systems. This evidence supports a role for cytosine methylation in maintaining transcriptional states.

The plant's need for developmental plasticity and environmental interaction suggests that plants extensively employ epigenetic regulatory strategies. Such strategies rely on heritable, often reversible, changes in access to the underlying genetic information, but not alteration of the primary nucleotide sequence. As one example, the alteration of DNA methylation is expected to perturb plant development significantly, provided that differential DNA methylation is an important component of epigenetic regulation in plants.

One paradigm linking DNA methylation and developmental regulation comes from work on the mouse, where average genome cytosine methylation levels in embryonic lineages drop sharply in the early cleavages following fertilization, then rise again around the time of implantation. In plants, a similar pattern has been observed in studies of DNA methylation content in pollen and post-embryonic tissue of varying age. Information from such studies indicates that there is a gradual rise in 5-methylcytosine levels in post-embryonic tissues

produced by meristems at positions further from the base of the plant (i.e., tissues of increasing age). Genetic studies of transposon systems in maize also demonstrate an age-dependent gradient of increasing epigenetic modification, which is correlated with DNA methylation.

Both biochemical and genetic approaches have been taken to alter DNA methylation in eucaryotic organisms. Methylation inhibitor treatments have induced developmental abnormalities in many plant species. Transgenic plants expressing antisense molecules specific for a native cytosine methyltransferase gene have been found to exhibit genomic hypomethylation, presumably due to the antisense interference with expression of the gene.

In another approach, mutants of *Arabidopsis thaliana* have been isolated, which show a decrease in DNA methylation (*ddm*) resulting in reduced nuclear 5-methylcytosine levels. The best characterized mutations define the *DDM1* gene. Homozygotes carrying recessive *ddm1* alleles contain 30% of the wild-type levels of 5-methylcytosine. The *ddm1* mutations do not map to the two known cytosine-DNA methyltransferase genes of *A. thaliana*, nor do they affect DNA methyltransferase activity detectable in nuclear extracts (Kakutani et al., *Nuc. Acids Res.* **23**: 130-137, 1995). In addition, *ddm1* mutations do not appear to affect the metabolism of the active methyl group donor, S-adenosylmethionine (Kakutani et al., 1995, *supra*).

For the foregoing reasons, the *DDM1* gene product is likely to be a novel component of the DNA methylation system, or involved in determining the cellular context (e.g., chromatin structure, subnuclear localization) of the methylation reaction. Consequently,

it would be a clear advance in the art of plant molecular and cellular biology to identify and isolate the *DDM1* gene and/or its encoded protein. Such a gene and protein would find utility for the purpose of modifying the methylation status of a selected genome and thereby altering one or more regulatory features of gene expression from that genome.

**SUMMARY OF THE INVENTION**

A novel gene, *DDM1*, and its encoded protein are provided in accordance with the present invention. The gene has been identified as a novel element of the DNA methylation system.

In one aspect of the invention, an isolated nucleic acid molecule comprising a gene located on *Arabidopsis thaliana* chromosome 5, lower arm, is provided. The gene occupies a segment of chromosome 5, lower arm, which is flanked on the centromeric side within 20 kilobases by a gene encoding a zinc-finger protein and on the telomeric side within 1 kilobase by a gene encoding a glutamic acid tRNA. Disruption of the gene is associated with DNA hypomethylation. The gene encodes a polypeptide of about 764 amino acids in length. The nucleotide sequence of the *DDM1* gene is set forth herein as SEQ ID NO:1 and its deduced amino acid sequence as SEQ ID NO:2. In SEQ ID NO:1, the regions of the gene that comprise coding sequence are indicated.

In another aspect of the invention, an isolated *DDM1* gene is provided, having a sequence selected from the group consisting of: (a) SEQ ID NO:1; (b) an allelic variant or natural mutant of SEQ ID NO:1; (c) a sequence hybridizing with part or all of SEQ ID NO:1 or its complement and encoding a polypeptide substantially the

same as part or all of a polypeptide encoded by SEQ ID NO:1; (d) a sequence encoding part or all of a polypeptide having amino acid SEQ ID NO:2; and (e) a sequence encoding part or all of a polypeptide contained in the cosmid clone C38, designated ATCC Accession No. 207208.

According to another aspect of the invention, a polypeptide is provided, which is produced by expression of an isolated nucleic acid molecule comprising part or all of an open reading frame of a gene located on *Arabidopsis thaliana* chromosome 5, lower arm, the gene occupying a segment of chromosome 5, lower arm, flanked on the centromeric side within 20 kilobases by a gene encoding a zinc-finger protein and on the telomeric side within 1 kilobase by a gene encoding a glutamic acid tRNA. This polypeptide preferably has the amino acid sequence of part or all of SEQ ID NO:2.

According to another aspect of the invention, an isolated protein encoded by an *Arabidopsis thaliana* gene is provided, which is a member of an SWI2/SNF2 family of polypeptides. Loss of function of the protein is associated with DNA hypomethylation. The protein is encoded by a gene located on *A. thaliana* chromosome 5, lower arm, centromerically flanked within 20 kilobases by a zinc finger-encoding gene and telomerically within one kilobase by a gene encoding a glutamic acid tRNA.

According to another aspect of the invention, a transgenic organism comprising the *DDM1* gene is provided. In one embodiment, the transgenic organism is a plant.

In other aspects of the invention, methods are provided for stabilizing fidelity of DNA methylation in an organism, which comprise transforming the organism with the *DDM1* gene. Methods are also provided for

reducing or eliminating gene silencing in a plant, or for inducing inbreeding depression in a plant, which comprise inhibiting or preventing expression of an endogenous *DDM1* gene of the plant.

These aspects of the invention, as well as other features and advantages of the invention, will be described in greater detail in the description and examples set forth below.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1.** Map-based isolation of the *A. thaliana DDM1* gene. A genetic map of the region of *A. thaliana* chromosome 5 containing the *DDM1* gene is shown at the top of the figure (see Example 1). The relative sizes of the genetic intervals were determined by the number of recombination breakpoints (rec bkpts) scored in a panel of recombinant lines containing cross-overs between flanking markers *yi* and *aba*. The regions represented in genomic clones T10D21 and C38 are denoted by the open boxes below the genetic map. The ~30 kb interval containing the *DDM1* gene, defined by the genetic markers A and D, is shown at the bottom of the figure. The number of recombination breakpoints scored between markers A - D and *ddm1-2* are indicated. The position of predicted coding regions in the interval are numbered and shown below the physical map. BAC, bacterial artificial chromosome; SuDH, succinate dehydrogenase structural gene.

**Figure 2.** *DDM1* gene structure and identification. **Fig. 2A:** The intron/exon structure of the *DDM1* gene. Protein-coding exons are shown as open boxes, with the start and stop codons indicated. Introns are depicted as thin lines. The position and nature of

four *ddm1* alleles are indicated above the exon/intron map. **Fig. 2B:** RT-PCR analysis of *ddm1-2* and wild-type *DDM1* transcripts. The approximate positions of oligonucleotide primers used in the analysis are shown below the map in Fig. 2A. Amplifications were done on either genomic templates (DNA), first-strand cDNA templates (+RT, plus reverse transcriptase), or mock-synthesized cDNA (-RT, minus reverse transcriptase). Amplified products were separated on a 3% agarose gel and visualized after ethidium bromide staining. Amplification from cDNA representing the properly spliced transcript resulted in a ~280 bp product. The nucleotide sequence of the ~220 bp product amplified from *ddm1-2* cDNA template indicated that the mutation leads to use of an alternate splice donor 56 bp upstream of the wild-type splice donor site.

**Figure 3.** The *A. thaliana DDM1* gene encodes a SWI2/SNF2-like protein. The deduced primary amino acid sequence of DDM1 (At DDM1) is aligned with two other SWI2/SNF2-like protein sequences, *Mus musculus* lymphocyte specific helicase (Mm LSH; SEQ ID NO:4) and human SNF2h (Hs SNF2h; SEQ ID NO:5). Sequence identities are indicated by black boxes and conservative changes are shaded. The positions of the eight signature motifs characteristic of SNF2 family proteins are indicated below the aligned sequences. Amino acid coordinates are indicated on the left; only the N-terminal 730 amino acids (of 1052 total) are shown for human SNF2h, though SEQ ID NO:5 shows the entire protein sequence. The deletion/frameshift caused by the *ddm1-2* allele occurs at amino acid 524. The *ddm1-6* frameshift occurs at amino acid 379, leading to translation of an additional 52 amino acids out of frame. The *ddm1-7* nonsense mutation

occurs at amino acid 549. Dashes indicate gaps introduced by the CLUSTAL W algorithm to maximize alignment (Thompson et al., Nucleic Acids Res. **22**: 4673-4680, 1994). The alignment was processed by BOXSHADE v. 3.21.

## DETAILED DESCRIPTION OF THE INVENTION

### I. Definitions

Various terms relating to the biological molecules of the present invention are used throughout the specification and claims.

With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule.

With respect to RNA molecules of the invention the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to protein, the term "isolated protein" or "isolated and purified protein" is sometimes

used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus define the differences. In the comparisons made in the present invention, the CLUSTLW program and parameters employed therein were utilized (Thompson et al., 1994, *supra*). However, equivalent alignments and similarity/identity assessments can be obtained through the use of any standard alignment software. For instance, the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program may also be used to compare sequence identity and similarity.

The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nature of the

protein (i.e. the structure, stability characteristics, substrate specificity and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

With respect to antibodies, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

With respect to oligonucleotides or other single-stranded nucleic acid molecules, the term "specifically hybridizing" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the regulatory sequences necessary for expression of the coding sequence are placed in a nucleic acid molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement other transcription control elements (e.g. enhancers) in an expression vector.

Transcriptional and translational control sequences are DNA regulatory sequences, such as

promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In particular, as used herein, the term "DNA transcriptional response element" refers to a DNA sequence specifically recognized for binding by a DNA binding protein characterized as a transcriptional regulator (either activator or suppressor).

The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA". Such a

nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

The term "reporter gene" refers to genetic sequences which may be operably linked to a promoter region forming a transgene, such that expression of the reporter gene coding region is regulated by the promoter and expression of the transgene is readily assayed.

The term "selectable marker gene" refers to a gene product that when expressed confers a selectable phenotype, such as antibiotic resistance, on a transformed cell or plant.

The term "DNA construct" is sometimes used herein to refer to genetic sequence used to transform plants and generate progeny transgenic plants. These constructs may be administered to plants in a viral or plasmid vector. Other methods of delivery such as Agrobacterium T-DNA mediated transformation and transformation using the biolistic process are also contemplated to be within the scope of the present invention. The transforming DNA may be prepared according to standard protocols such as those set forth in "Current Protocols in Molecular Biology", eds. Frederick M. Ausubel et al., John Wiley & Sons, 1995.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA construct when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and plant cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells

through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

## II. Description of *DDM1* and its Encoded Polyeptide

In accordance with the present invention, a novel gene, *DDM1*, has been isolated from the flowering plant *Arabidopsis thaliana*. Through analysis of mutant plants, this gene has been identified as important for the maintenance of proper genomic cytosine methylation, and its function appears to be necessary to maintain gene silencing. Biochemical and molecular genetic results indicate that *DDM1* encodes a novel component of the DNA methylation machinery.

We have isolated the *DDM1* gene from *A. thaliana* using a map-based cloning approach, which is described in detail in Example 1 and shown in Figure 1. Briefly, the *DDM1* gene was initially localized to the bottom of the lower arm of chromosome 5 by reference to molecular markers segregating in an F2 family (parental cross: Columbia *ddm1/ddm1* X Landsberg erecta *DDM1/DDM1*). Next, recombination breakpoints in the region surrounding a *ddm1* mutation were isolated by collecting cross-over chromosomes by reference to flanking genetic markers. The recombination breakpoints delimited a region of approximately 30 kilobases. Cloned DNA corresponding to this genomic region was isolated by subcloning DNA from a

bacterial artificial chromosome (BAC) containing molecular markers mapping both proximal and distal to the *ddm1* marker. The nucleotide sequence of a single cosmid subclone encompassing the 30 kb region was determined to identify six candidate genes, in addition to a tRNA gene and a previously identified succinate dehydrogenase structural gene.

The search for the *DDM1* gene focused on predicted genes 5 and 6, which fell in the center of the genetic interval defined by recombination breakpoints with the *ddm1-2* marker. The *DDM1* gene was identified as predicted gene 6 based on DNA sequence alterations in four *ddm1* alleles (Figure 2). The EMS-generated *ddm1-2* mutation is a G to A transition in the splice donor site of intron 11 that forces the use of an alternate splice donor site 56 bp upstream in exon 11 (Fig. 2B). The splicing defect leads to a deletion, a frameshift and premature translation termination upstream of predicted functional domains. The fast neutron-generated *ddm1-5* (previously named *som8*; Mittelsten Scheid, O., Afsar, K. & Paszkowski, J. *Proc. Natl. Acad. Sci. USA* **95**: 632-637, 1998).) allele contains an 82 bp insertion (1 bp deleted and replaced with 83 bp) in the second protein-coding exon, leading to an in-frame stop after 30 codons (15 wild-type codons plus 15 codons from the insertion). Premature translation termination is also predicted to result from two additional fast neutron alleles: *ddm1-6* (*som4*) corresponds to a frameshift (1 bp deletion) in exon 7 and *ddm1-7* (*som5*) is a nonsense mutation in exon 12. All four characterized *ddm1* alleles are expected to destroy or severely reduce gene function.

The wild-type *DDM1* gene encodes a predicted protein of 764 amino acids with a high degree of

similarity to SWI2/SNF2-like proteins. Members of the SWI2/SNF2 family are involved in various functions, including transcriptional co-activation, transcriptional co-repression, chromatin assembly and DNA repair. Underlying these apparently diverse activities is the modification or disruption of protein-DNA interactions by multi-protein complexes which contain SWI2/SNF2-like components. Figure 3 shows an alignment among the deduced amino acid sequences of *A. thaliana* DDM1 and two mammalian members of the SNF2 family, human SNF2h (SEQ ID NO:4; Arihara, T. *et al.*, *Cytogenet. Cell Genet.* **81**, 191-193, 1998) and murine LSH (SEQ ID NO:5; lymphocyte specific helicase, LSH; Jarvis, C.D. *et al.* *Gene* **169**, 203-207, 1996). DDM1 contains the eight sequence motifs diagnostic of SWI2/SNF2 family members (Bork, P. & Koonin, E.V. *Nucleic Acids Res.* **21**, 751-752, 1993). *A. thaliana* DDM1 and human SNF2h share 45 percent identity over the approximately 470 amino acid region comprising the signature motifs. Over a similar region, *A. thaliana* DDM1 and murine LSH display approximately 50 percent identity, omitting the 47 residues (amino acids 276-322) apparently unique to LSH. Initial molecular phylogenetic analysis placed DDM1 in a small subfamily, within the SNF2 family, which contains proteins of unknown function, including murine LSH (Eisen, J.A. *et al.* *Nucleic Acids Res.* **23**, 2715-2723, 1995). The proteins of known function most closely related to DDM1 are involved in chromatin remodeling and are grouped in the SNF2L/ISWI subfamily (Eisen *et al.*, 1995, *supra*).

Without intending to be bound by any particular mechanism for the functionality of the *DDM1* gene product, analysis of the foregoing data indicates that the *DDM1* protein functions in the DNA methylation system by

affecting chromatin structure. Two general models for the *DDM1* action are envisioned. The *DDM1* protein may function as a transcriptional co-activator, similar to many SWI2/SNF2-like proteins, to increase the expression of a component of the DNA methylation system. *DDM1* does not affect DNA methyltransferase expression directly because *ddm1* mutant extracts contain wild-type methyltransferase activity (Kakutani et al., 1995, *supra*). However, an unidentified positive effector of DNA methylation may be a target. Alternatively, wild-type *DDM1* function may change chromatin structure to direct certain sequences to the methylation machinery or to facilitate the methylation of genomic substrates. The recently discovered interplay between cytosine methylation and histone acetylation , and the association of SWI2/SNF2-like proteins and histone deacetylases in chromatin remodeling complexes, makes it plausible that *DDM1* affects DNA methylation through modulation of histone modification or another aspect of chromatin structure. Another possibility is that *DDM1* plays a more direct role as a part of a nucleosome remodeling complex that increases the accessibility of the DNA methyltransferase to the hemimethylated substrates in newly replicated chromatin. The latter model is particularly attractive because it predicts that *ddm1* mutations will preferentially hypomethylate genomic sequences packaged in highly condensed chromatin while causing slower loss of methylation in more accessible sequences, consistent with the observed hypomethylation specificity of *ddm1* mutations. The isolation of the *Arabidopsis DDM1* gene in accordance with the present invention points to the importance of chromatin dynamics in the maintenance of cytosine methylation patterns and

identifies a novel component of the eukaryotic DNA methylation pathway.

A number of applications are contemplated for the novel gene of the invention and its encoded protein, and the discovery of the involvement of a *SWI2/SNF2*-like gene in the eucaryotic DNA methylation system. Such applications are described in greater detail below.

Although the *DDM1* genomic clone from *Arabidopsis thaliana* is described and exemplified herein, this invention is intended to encompass nucleic acid sequences and proteins from other organisms, including plants, yeast, insects and mammals, that are sufficiently similar to be used instead of the *Arabidopsis DDM1* nucleic acid and proteins for the purposes described below. These include, but are not limited to, allelic variants and natural mutants of SEQ ID NO:1, which are likely to be found in different species of plants or varieties of *Arabidopsis*. Because such variants are expected to possess certain differences in nucleotide and amino acid sequence, this invention provides an isolated *DDM1* nucleic acid molecule having at least about 60% (preferably 70% and more preferably over 80%) sequence homology in the coding regions with the nucleotide sequence set forth as SEQ ID NO:1 (and, most preferably, specifically comprising the coding region of SEQ ID NO:1). This invention also provides isolated polypeptide products of the open reading frames of SEQ ID NO:1, having at least about 60% (preferably 70% or 80% or greater) sequence homology with the amino acid sequences of SEQ ID NO:2. Because of the natural sequence variation likely to exist among *DDM1* genes, one skilled in the art would expect to find up to about 30-40% nucleotide sequence variation, while still maintaining

the unique properties of the *DDM1* gene and encoded polypeptide of the present invention. Such an expectation is due in part to the degeneracy of the genetic code, as well as to the known evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the encoded protein. Accordingly, such variants are considered substantially the same as one another and are included within the scope of the present invention.

The following description sets forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (1999) (hereinafter "Ausubel et al.") are used.

## A. Preparation of *DDM1* Nucleic Acid Molecules, encoded Polypeptides and Antibodies Specific for the Polypeptides

### 1. Nucleic Acid Molecules

*DDM1* nucleic acid molecules of the invention may be prepared by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art.

The availability of nucleotide sequence information, such as the cDNA having SEQ ID NO:1, enables preparation of an isolated nucleic acid molecule of the

invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramadite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC). Long, double-stranded polynucleotides, such as a DNA molecule of the present invention, must be synthesized in stages, due to the size limitations inherent in current oligonucleotide synthetic methods. Thus, for example, a long double-stranded molecule may be synthesized as several smaller segments of appropriate complementarity. Complementary segments thus produced may be annealed such that each segment possesses appropriate cohesive termini for attachment of an adjacent segment. Adjacent segments may be ligated by annealing cohesive termini in the presence of DNA ligase to construct an entire long double-stranded molecule. A synthetic DNA molecule so constructed may then be cloned and amplified in an appropriate vector.

*DDM1* genes also may be isolated from appropriate biological sources using methods known in the art. In the exemplary embodiment of the invention, the *A. thaliana DDM1* clone was isolated from a BAC genomic library of *A. thaliana* In alternative embodiments, cDNA clones of *DDM1* may be isolated. A preferred means for isolating *DDM1* genes is PCR amplification using genomic templates and *DDM1*-specific primers.

In accordance with the present invention, nucleic acids having the appropriate level sequence homology with part or all the coding regions of SEQ ID NO:1 may be identified by using hybridization and washing conditions of appropriate stringency. For example,

hybridizations may be performed, according to the method of Sambrook et al., using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 2X SSC and 0.1% SDS; (4) 2 hours at 45-55°in 2X SSC and 0.1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989):

$T_m = 81.5°C + 16.6\text{Log}\,[Na+] + 0.41(\%\ G+C) - 0.63\ (\%\ \text{formamide}) - 600/\#\text{bp in duplex}$

As an illustration of the above formula, using [N+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the $T_m$ is 57°C. The $T_m$ of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such a sequence would be considered substantially homologous to the sequences of the present invention.

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid cloning/expression vector, such as pGEM-T (Promega Biotech, Madison, WI) or pBluescript (Stratagene, La Jolla, CA), either of which is propagated in a suitable

*E. coli* host cell.

*DDM1* nucleic acid molecules of the invention include cDNA, genomic DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of the DNA having SEQ ID NO:1. Such oligonucleotides are useful as probes for detecting *DDM1* genes or mRNA in test samples, e.g. by PCR amplification, or for the positive or negative regulation of expression of *DDM1* genes at or before translation of the mRNA into proteins.

The *DDM1* promoter and other expression regulatory sequences for *DDM1* are also expected to be useful in connection with the present invention. SEQ ID NO:1 shows about 550 bp of sequence upstream from the beginning of the coding region, which should contain such expression regulatory sequences. In addition, SEQ ID NO:3 constitutes about 5 kbp of additional upstream sequence, which should contain other regulatory sequences, such as enhancer elements.

## 2. Proteins

Polypeptides encoded by *DDM1* nucleic acids of the invention may be prepared in a variety of ways, according to known methods. If produced *in situ* the polypeptides may be purified from appropriate sources, e.g., plant parts.

Alternatively, the availability of nucleic acid molecules encoding the polypeptides enables production of the proteins using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into

an appropriate *in vitro* transcription vector, such a pSP64 or pSP65 for *in vitro* transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. *In vitro* transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland.

According to a preferred embodiment, larger quantities of *DDM1*-encoded polypeptide may be produced by expression in a suitable procaryotic or eucaryotic system. For example, part or all of a DNA molecule, such as the coding portion of SEQ ID NO:1, may be inserted into a plasmid vector adapted for expression in a bacterial cell (such as *E. coli*) or a yeast cell (such as *Saccharomyces cerevisiae*), or into a baculovirus vector for expression in an insect cell. Such vectors comprise the regulatory elements necessary for expression of the DNA in the host cell, positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

The DDM1 polypeptide produced by gene expression in a recombinant procaryotic or eucyarotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used by skilled practitioners.

The *DDM1*-encoded polypeptides of the invention, prepared by the aforementioned methods, may be analyzed according to standard procedures. Methods for analyzing the functional activity are available. For instance, DNA methylation levels are detectable by known methods. Alternatively, the function of the *DDM1* gene product as part of a chromatin remodeling machine permits the use of *in vitro* assays for chromatin remodeling, which are known in the art (e.g., B.R. Cairns, *Trends in Biochem.* **23**: 20-25, 1998).

The present invention also provides antibodies capable of immunospecifically binding to polypeptides of the invention. Polyclonal or monoclonal antibodies directed toward the polypeptide encoded by *DDM1* may be prepared according to standard methods. Monoclonal antibodies may be prepared according to general methods of Köhler and Milstein, following standard protocols. In a preferred embodiment, antibodies are prepared, which react immunospecifically with various epitopes of the *DDM1*-encoded polypeptides.

## B. Uses of *DDM1* Nucleic Acids, Encoded Proteins and Antibodies

### 1. *DDM1* Nucleic Acids

*DDM1* nucleic acids may be used for a variety of purposes in accordance with the present invention. The DNA, RNA, or fragments thereof may be used as probes to detect the presence of and/or expression of *DDM1* genes. Methods in which *DDM1* nucleic acids may be utilized as probes for such assays include, but are not limited to: (1) *in situ* hybridization; (2) Southern hybridization (3) northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR).

The *DDM1* nucleic acids of the invention may also be utilized as probes to identify related genes from

other species, including but not limited to, plants, yeast, insects and mammals, including humans. As is well known in the art and described above, hybridization stringencies may be adjusted to allow hybridization of nucleic acid probes with complementary sequences of varying degrees of homology. Thus, *DDM1* nucleic acids may be used to advantage to identify and characterize other genes of varying degrees of relation to the exemplary coding sequence of SEQ ID NO:1, thereby enabling further characterization of this family of genes. Additionally, they may be used to identify genes encoding proteins that interact with protein encoded by *DDM1* (e.g., by the "interaction trap" technique).

As discussed above and in greater detail in Example 1, the similarity among plant *DDM1* and its *SWI2/SNF2* counterparts in yeast, *Drosophila* and mammals indicates that the functional aspects of these proteins will also be conserved. Thus, *DDM1* is expected to play an important role in DNA methylation and resultant down-regulation of gene expression. Plants engineered to over-express *DDM1* can be expected to have improved fidelity of the DNA methylation system. The evidence suggests that loss of DDM1 function leads to reduction in the efficiency of maintenance methylation due to reduced accessibility of the methyltransferase enzyme to the substrate. Hence, excess DDM1 function could lead to an increase in the fidelity of the inheritance of DNA methylation thereby reducing the occurrence of spurious methylation mistakes which could compromise the organism's viability or fecundity. In fact, there are experimental data demonstrating that loss of DDM1 function leads to stochastic hypermethylation, and epigenetic lesion formation, as well. For these reasons, *DDM1* overexpression lines are expected to have useful properties.

Transgenic plants expressing the *DDM1* gene or antisense nucleotides can be generated using standard plant transformation methods known to those skilled in the art. These include, but are not limited to, *Agrobacterium* vectors, PEG treatment of protoplasts, biolistic DNA delivery, UV laser microbeam, gemini virus vectors, calcium phosphate treatment of protoplasts, electroporation of isolated protoplasts, agitation of cell suspensions with microbeads coated with the transforming DNA, direct DNA uptake, liposome-mediated DNA uptake, and the like. Such methods have been published in the art. See, e.g., Methods for Plant Molecular Biology (Weissbach & Weissbach, eds., 1988); Methods in Plant Molecular Biology (Schuler & Zielinski, eds., 1989); Plant Molecular Biology Manual (Gelvin, Schilperoort, Verma, eds., 1993); and Methods in Plant Molecular Biology - A Laboratory Manual (Maliga, Klessig, Cashmore, Gruissem & Varner, eds., 1994).

The method of transformation depends upon the plant to be transformed. The biolistic DNA delivery method is useful for nuclear transformation. In another embodiment of the invention, *Agrobacterium* vectors are used to advantage for efficient transformation of plant nuclei.

In a preferred embodiment, the gene is introduced into plant nuclei in *Agrobacterium* binary vectors. Such vectors include, but are not limited to, BIN19 (Bevan, 1984) and derivatives thereof, the pBI vector series (Jefferson et al., 1987), and binary vectors pGA482 and pGA492 (An, 1986).

The *DDM1* gene may be placed under a powerful constitutive promoter, such as the Cauliflower Mosaic Virus (CaMV) 35S promoter or the figwort mosaic virus 35S promoter. Transgenic plants expressing the *DDM1* gene under an inducible promoter (either its own promoter or a

heterologous promoter) are also contemplated to be within the scope of the present invention. Inducible plant promoters include the tetracycline repressor/operator controlled promoter.

Using an *Agrobacterium* binary vector system for transformation, the *DDM1* coding region, under control of a constitutive or inducible promoter as described above, is linked to a nuclear drug resistance marker, such as kanamycin resistance. *Agrobacterium*-mediated transformation of plant nuclei is accomplished according to the following procedure:

(1) the gene is inserted into the selected *Agrobacterium* binary vector;

(2) transformation is accomplished by co-cultivation of plant tissue (e.g., leaf discs) with a suspension of recombinant *Agrobacterium*, followed by incubation (e.g., two days) on growth medium in the absence of the drug used as the selective medium (see, e.g., Horsch et al. 1985);

(3) plant tissue is then transferred onto the selective medium to identify transformed tissue; and

(4) identified transformants are regenerated to intact plants.

It should be recognized that the amount of expression, as well as the tissue specificity of expression of the *DDM1* gene in transformed plants can vary depending on the position of their insertion into the nuclear genome. Such position effects are well known in the art. For this reason, several nuclear transformants should be regenerated and tested for expression of the transgene.

In some instances, it may be desirable to down-regulate or inhibit expression of endogenous *DDM1* in plants possessing the gene. One clear benefit to engineering a reduction of DDM1 function is to reduce

gene (including transgene) silencing. Plant lines with reduced or absent DDM1 function are expected to be viable based on results obtained with *Arabidopsis*. Further, it has been shown that gene silencing is suppressed in *ddm1* *Arabidopsis* lines (Jeddeloh et al., *Genes Devel.* **12**:1714-1725, 1998). There are two other beneficial characteristics of *DDM1* deficient plant lines. First, alteration in DNA methylation leads to changes in flowering time, and as such, is a potentially powerful tool for manipulating plant development. (See, e.g., Richards, *Trends in Genetics* **13**: 319-323, 1998), Second, *ddm1* mutant lines exhibit inbreeding depression (a reduction in vigor after inbreeding) (Richards, Trends in Genetics, 1998, *supra*), a characteristic which may be desirable to include in situations where proprietary germplasms in hybrid plants are at risk of unauthorized use. For instance, a genetically engineered hybrid (containing one or more useful transgenes) could be further engineered to down-regulate endogenous *DDM1* expression. Unauthorized inbreeding of such lines would be discouraged because the progeny of such lines would lack vigor.

To achieve the aforementioned benefits associated with reduced gene expression, *DDM1* nucleic acid molecules, or fragments thereof, may also be utilized to control the production of *DDM1*-encoded proteins. In one embodiment, full-length *DDM1* antisense molecules or antisense oligonucleotides, targeted to specific regions of *DDM1*-encoded RNA that are critical for translation, are used. The use of antisense molecules to decrease expression levels of a pre-determined gene is known in the art. In a preferred embodiment, antisense molecules are provided *in situ* by transforming plant cells with a DNA construct which, upon

transcription, produces the antisense sequences. Such constructs can be designed to produce full-length or partial antisense sequences.

In another embodiment, overexpression of *DDM1* is induced to generate a co-suppression effect. This excess expression serves to promote down-regulation of both endogenous and exogenous *DDM1* genes.

Optionally, transgenic plants can be created containing mutations in the region encoding the active site of *DDM1*. This embodiment may be preferred in certain instances.

From the foregoing discussion, it can be seen that *DDM1* and its homologs will be useful for introducing alterations in gene expression in an organism, for a variety of purposes. As described above, for instance, the *Arabidopsis DDM1* gene can be used to isolate mutants or engineer organisms that express reduced function of *DDM1* orthologs. Based on results in *Arabidopsis*, such mutants or engineered organisms are expected to be viable and display valuable characteristics, such as inbreeding depression and a reduction in gene silencing. In addition, we anticipate that dysfunction in human *DDM1* orthologs may contribute to diseases that involve alterations in DNA methylation, including cancer (Baylin, S.B. et al., *Adv. Cancer Res.* **72**: 141-196, 1998) and immunodeficiency/ chromosome instability/facial anomalies syndrome (ICF) (Smeets, D.F.C.M. et al., *Hum. Genet.* **94**: 240-246, 1994).

## 2. DDM1 Proteins and Antibodies

Purified *DDM1*-encoded proteins, or fragments thereof, may be used to produce polyclonal or monoclonal antibodies which also may serve as sensitive detection reagents for the presence and accumulation of*DDM1*-encoded

protein in cultured cells or tissues and in intact organisms. Recombinant techniques enable expression of fusion proteins containing part or all of the *DDM1*-encoded protein. The full length protein or fragments of the protein may be used to advantage to generate an array of monoclonal or polyclonal antibodies specific for various epitopes of the protein, thereby providing even greater sensitivity for detection of the protein in cells or tissue.

DDM1 gene products also may be useful as pharmaceutical agents if it is determined that DDM1 loss of function plays a role in carcinogenesis, as mentioned above. The gene products could be administered as replacement therapy for persons having neoplasias associated with DDM1 loss of function.

Polyclonal or monoclonal antibodies immunologically specific for *DDM1*-encoded proteins may be used in a variety of assays designed to detect and quantitate the protein. Such assays include, but are not limited to: (1) flow cytometric analysis; (2) immunochemical localization in cultured cells or tissues; and (3) immunoblot analysis (e.g., dot blot, Western blot) of extracts from various cells and tissues.

Polyclonal or monoclonal antibodies that immunospecifically interact with the polypeptide encoded by *DDM1* can be utilized for identifying and purifying such proteins. For example, antibodies may be utilized for affinity separation of proteins with which they immunospecifically interact. Antibodies may also be used to immunoprecipitate proteins from a sample containing a mixture of proteins and other biological molecules.

The following specific examples are provided to illustrate embodiments of the invention. They are not intended to limit the scope of the invention in any way.

## EXAMPLE 1
## Map-Based Isolation of the *Arabidopsis thaliana DDM1* Gene

**Construction of recombination breakpoint lines.** The recombination breakpoint lines were assembled in the F3 generation from a parental cross between *YI DDM1 ABA/YI ddm1-2 ABA* (Columbia strain (Col)) and *yi DDM1 aba/yi DDM1 aba* (Landsberg erecta strain (La er)). The recessive *yi* mutation leads to a yellow inflorescence. The recessive *aba* mutation causes a defect in abscisic acid biosynthesis and a wilting phenotype. Information on genetic markers and the *A. thaliana* genetic map can be found at: http://genome-www.stanford.edu/Arabidopsis/. Selfed seeds from F1 *YI ddm1-2 ABA/yi DDM1 aba* plants were collected and 135 F2 recombinants (yi ABA, yellow inflorescence, non-wilting; or YI aba: green inflorescence, wilting) were identified. Selfed seeds from 111 of the 135 recombinant F2 individuals were planted to generate F3 tissue for genomic DNA preparation. The genotype at the *DDM1* locus was scored in the F3 generation by Southern blot analysis using methylation-sensitive endonucleases as described previously (Vongs, A., Kakutani, T., Martienssen, R.A. & Richards, E.J. , *Science* **260**: 1926-1928, 1993).

**Molecular markers.** Two of the molecular markers shown in Figure 1 were available from the *Arabidopsis* research community: g4510 (*Arabidopsis* Biological Resource Center (ABRC) stock# CD2-38) and mi335 (ABRC stock# CD3-288). The remainder of the molecular markers shown in Figure 1 were developed in accordance with the present invention. sT10D21Bam is an

insert end subclone of the BAC (bacterial artificial chromosome) clone T10D21 constructed by complete cleavage with *Bam*HI and recircularization. sT10D21Bam recognizes a Col/La er *Pst*I RFLP (restriction fragment length polymorphism). Molecular marker A is an *Xba*I Col/La er RFLP marker recognized by a 5.7 kb *Hind*III fragment of the C38 cosmid insert. Marker B is a *Rsa*I Col/La er CAPS marker (Koneiczny & Ausubel, Plant J. **4**: 403-410, 1993) (forward primer: 5'-TCAAGGAGATGATTCGGGCGT-3', SEQ ID NO: 6; reverse primer: 5'-AAAGGACCCATTTACAGAACAC-3', SEQ ID NO:7). The remaining markers, C and D, correspond to RFLP's (*Bcl*I and *Pst*I, respectively) recognized by the succinate dehydrogenase cDNA clone, 105N23T7 (ABRC stock# 105N23T7).

**Genomic library construction and screening.** We screened the available *A. thaliana* BAC genomic libraries by standard colony hybridization techniques using radiolabeled 105N23T7 insert as a probe. The clone we subsequently focused upon, T10D21, came from the Texas A&M University BAC library (Choi et al., *Weeds World* **2**: 17-20, 1995). To facilitate subsequent analysis, we cloned *Sau*3AI partially digested fragments from the T10D21 insert into the *Bam*HI site of SuperCos (Stratagene). We chose to further characterize one member of the resulting cosmid sublibrary, C38, which contained genetic markers that flanked *ddm1-2*. The C38 cosmid was submitted on April 20, 1999, under the provisions of the Budapest Treaty, with the American Type Culture Collection (Manassas VA), and assigned ATCC Accession No. 207208.

## EXAMPLE 2
## *DDM1* Gene Structure and Identification; Sequence Determination of *DDM1* Gene

**DNA sequence determination.** C38 cosmid (~45 kb) DNA, prepared using Qiagen columns and protocols, was sonicated and 1-2 kb fragments isolated from a low-melting temperature agarose gel. The size-selected DNA was cloned into the *Sma*I site of a M13mp18 vector to generate a shotgun library suitable for DNA sequence determination. Single-stranded substrates were prepared and sequenced using conventional dye-terminator cycle sequencing protocols (Perkin-Elmer) on either an ABI 373 or ABI 377 automated DNA sequencer. The DNA sequence of the *ddm1* alleles was determined using PCR-amplified templates and oligonucleotide primers dispersed throughout the *DDM1* gene. Sequence assembly and analysis were accomplished using Phred/Phrap/Consed (http://www.mbt.washington.edu/) and DNASTAR software suites.

**RT-PCR cDNA analysis.** *DDM1* gene structure was determined by analysis of the genomic DNA sequence and the nucleotide sequence of RT-PCR (reverse transcription-polymerase chain reaction) products encompassing the coding region. *DDM1* and *ddm1-2* transcripts were analyzed by RT-PCR as follows. Total RNA was prepared using the Qiagen RNeasy™ protocol. Poly(A)+ transcripts were collected on oligo-d$(T)_{25}$ magnetic Dynabeads (Dynal) and first-strand cDNA synthesis performed following Dynal protocols using Stratascript (Stratagene) reverse transcriptase. Aliquots of the bead-immobilized first-strand cDNA library were used as templates for PCR amplification using KlenTaqI polymerase (Clontech). The following oligonucleotide primers were used for the RT-PCR experiment shown in Fig. 2*b*: forward, 5'-GCTGGAAGGGAAAGCTTAACAACC-3' (SEQ ID NO:8); reverse,

5'-ACACTGCCATCGATTCTGCAAACC-3' (SEQ ID NO:9).

**GenBank accession numbers and SEQ ID NOS.**

*Arabidopsis DDM1* genomic DNA sequence: SEQ ID NO:1;

*Arabidopsis* DDM1 deduced amino acid sequence: SEQ ID NO:2;

*Arabidopsis DDM1* 5' upstream genomic DNA sequence: SEQ ID NO:3;

*Mus musculus* lymphocyte specific helicase (LSH); Genbank Accession No. AAB08015; SEQ ID NO:4;

*Homo sapiens* SNF2h; Genbank Accession No. AB010882; SEQ ID NO:5;

succinate dehydrogenase cDNA 105N23T7, T22529;

primers: SEQ ID NOS: 6-9.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

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Jeffrey A. Jeddeloh

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65                  70                  75                  80
Glu Glu Lys Ala Asn Asn Ala Gly Ser Ala Val Ala Pro Asn Leu Asn
                85                  90                  95
Glu Thr Gln Phe Thr Lys Leu Asp Glu Leu Leu Thr Gln Thr Gln Leu
            100                 105                 110
Tyr Ser Glu Phe Leu Leu Glu Lys Met Glu Asp Ile Thr Ile Asn Gly
        115                 120                 125
Ile Glu Ser Glu Ser Gln Lys Ala Glu Pro Glu Lys Thr Gly Arg Gly
    130                 135                 140
Arg Lys Arg Lys Ala Ala Ser Gln Tyr Asn Asn Thr Lys Ala Lys Arg
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Ala Val Ala Ala Met Ile Ser Arg Ser Lys Glu Asp Gly Glu Thr Ile
                165                 170                 175
Asn Ser Asp Leu Thr Glu Glu Glu Thr Val Ile Lys Leu Gln Asn Glu
            180                 185                 190
Leu Cys Pro Leu Leu Thr Gly Gly Gln Leu Lys Ser Tyr Gln Leu Lys
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Gly Val Lys Trp Leu Ile Ser Leu Trp Gln Asn Gly Leu Asn Gly Ile
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Leu Ala Asp Gln Met Gly Leu Gly Lys Thr Ile Gln Thr Ile Gly Phe
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Ala Pro Leu Ser Thr Leu Ser Asn Trp Phe Asn Glu Ile Ala Arg Phe
            260                 265                 270
Thr Pro Ser Ile Asn Ala Ile Ile Tyr His Gly Asp Lys Asn Gln Arg
        275                 280                 285
Asp Glu Leu Arg Arg Lys His Met Pro Lys Thr Val Gly Pro Lys Phe
    290                 295                 300
Pro Ile Val Ile Thr Ser Tyr Glu Val Ala Met Asn Asp Ala Lys Arg
305                 310                 315                 320
Ile Leu Arg His Tyr Pro Trp Lys Tyr Val Val Ile Asp Glu Gly His
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Arg Leu Lys Asn His Lys Cys Lys Leu Leu Arg Glu Leu Lys His Leu
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Lys Met Asp Asn Lys Leu Leu Leu Thr Gly Thr Pro Leu Gln Asn Asn
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Cys Asn His Pro Asp Leu Leu Gln Gly Gln Ile Asp Gly Ser Tyr Leu
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Lys Pro Val His Val Tyr Arg Leu Ser Thr Ala Gln Ser Ile Glu Thr
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Ile Gly Gln Gly Gln Phe His Gln Glu Arg Ala Lys Ser Ser Thr Pro
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Leu Glu Glu Glu Asp Ile Leu Ala Leu Leu Lys Glu Asp Glu Thr Ala
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Glu Asp Lys Leu Ile Gln Thr Asp Ile Ser Asp Ala Asp Leu Asp Arg
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Pro Asn Trp Met Ala Glu Phe Lys Arg Phe Thr Pro Glu Ile Pro Thr
    50                  55                  60
Leu Leu Tyr His Gly Thr Arg Glu Asp Arg Arg Lys Leu Val Lys Asn
65                  70                  75                  80
Ile His Lys Arg Gln Gly Thr Leu Gln Ile His Pro Val Val Val Thr
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Ser Phe Glu Ile Ala Met Arg Asp Gln Asn Ala Leu Gln His Cys Tyr
            100                 105                 110
Trp Lys Tyr Leu Ile Val Asp Glu Gly His Arg Ile Lys Asn Met Lys
        115                 120                 125
Cys Arg Leu Ile Arg Glu Leu Lys Arg Phe Asn Ala Asp Asn Lys Leu
    130                 135                 140
Leu Leu Thr Gly Thr Pro Leu Gln Asn Asn Leu Ser Glu Leu Trp Ser
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Leu Leu Asn Phe Leu Leu Pro Asp Val Phe Asp Asp Leu Lys Ser Phe
                165                 170                 175
Glu Ser Trp Phe Asp Ile Thr Ser Leu Ser Glu Thr Ala Glu Asp Ile
            180                 185                 190
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        195                 200                 205
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225                 230                 235                 240
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Phe Gly Ser Cys Glu Lys Glu Thr Val Glu Leu Ser Pro Thr Gly Arg
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Pro Lys Arg Arg Ser Arg Lys Ser Ile Asn Tyr Ser Glu Leu Asp Gln
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Phe Pro Ser Glu Leu Glu Lys Leu Ile Ser Gln Ile Gln Pro Glu Val
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305                 310                 315                 320
Val Asn Leu Lys Leu Arg Asn Ile Met Met Leu Leu Arg Lys Cys Cys
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Asn His Pro Tyr Met Ile Glu Tyr Pro Ile Asp Pro Val Thr Gln Glu
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His Leu Arg Asn Phe Ile Phe Ser Arg Leu Asp Gly Ser Met Ser Tyr
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Ser Glu Arg Glu Lys Asn Ile Tyr Ser Phe Asn Thr Asp Pro Asp Val
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Phe Leu Phe Leu Val Ser Thr Arg Ala Gly Gly Leu Gly Ile Asn Leu
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<400> 5

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Ala Ser Ala Gly Pro Ala Asp Ala Glu Met Glu Glu Ile Phe Asp Asp
    50                  55                  60
Ala Ser Pro Gly Lys Gln Lys Glu Ile Gln Glu Pro Asp Pro Thr Tyr
65                  70                  75                  80
Glu Glu Lys Met Gln Thr Asp Arg Ala Asn Arg Phe Glu Tyr Leu Leu
                85                  90                  95
Lys Gln Thr Glu Leu Phe Ala His Phe Ile Gln Pro Ala Ala Gln Lys
            100                 105                 110
Thr Pro Thr Ser Pro Leu Lys Met Lys Pro Gly Arg Pro Arg Ile Lys
        115                 120                 125
Lys Asp Glu Lys Gln Asn Leu Leu Ser Val Gly Asp Tyr Arg His Arg
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**We claim:**

1. An isolated nucleic acid molecule comprising a gene located on *Arabidopsis thaliana* chromosome 5, lower arm, said gene occupying a segment of said chromosome 5, lower arm, flanked on the centromeric side within 20 kilobases by a gene encoding a zinc-finger protein and on the telomeric side within 1 kilobase by a gene encoding a glutamic acid tRNA, the disruption of said gene being associated with DNA hypomethylation.

2. The nucleic acid molecule of claim 1, wherein said gene is composed of exons that form an open reading frame having a sequence that encodes a polypeptide about 750-850 amino acids in length.

3. A cDNA molecule comprising the exons of the nucleic acid molecule of claim 2.

4. The nucleic acid molecule of claim 2, wherein said open reading frame encodes an amino acid sequence substantially the same as SEQ ID NO:2.

5. The nucleic acid molecule of claim 4, wherein said open reading frame encodes amino acid SEQ ID NO:2.

6. The nucleic acid molecule of claim 5, which comprises an open reading frame of SEQ ID NO:1.

7. A recombinant DNA molecule, comprising a vector having an insert that includes the nucleic acid molecule of claim 1.

8. The recombinant DNA molecule of claim 7,

which is cosmid C38, ATCC Accession No. 207208.

9. An oligonucleotide between about 10 and 100 nucleotides in length, which specifically hybridizes with a portion of the nucleic acid molecule of claim 1.

10. An isolated nucleic acid molecule which is a gene, the disruption of which is associated with DNA hypomethylation, having a sequence selected from the group consisting of:

a) SEQ ID NO:1;

b) an allelic variant or natural mutant of SEQ ID NO:1;

c) a sequence hybridizing with part or all of SEQ ID NO:1 or its complement and encoding a polypeptide substantially the same as part or all of a polypeptide encoded by SEQ ID NO:1;

d) a sequence encoding part or all of a polypeptide having amino acid SEQ ID NO:2; and

e) a sequence encoding part or all of a polypeptide contained in the cosmid clone C38, designated ATCC Accession No. 207208.

11. A polypeptide produced by expression of an isolated nucleic acid molecule comprising part or all of an open reading frame of a gene located on *Arabidopsis thaliana* chromosome 5, lower arm, said gene occupying a segment of said chromosome 5, lower arm, flanked on the centromeric side within 20 kilobases by a gene encoding a zinc-finger protein and on the telomeric side within 1 kilobase by a gene encoding a glutamic acid tRNA, the disruption of said gene being associated with DNA hypomethylation.

12. The polypeptide of claim 11, produced by

expression of a sequence selected from the group consisting of:

a) SEQ ID NO:1;

b) an allelic variant or natural mutant of SEQ ID NO:1;

c) a sequence hybridizing with part or all of SEQ ID NO:1 or its complement and encoding a polypeptide substantially the same as part or all of a polypeptide encoded by SEQ ID NO:1;

d) a sequence encoding part or all of a polypeptide having amino acid SEQ ID NO:2; and

e) a sequence encoding part or all of a polypeptide contained in the clone designated ATCC Accession No. 207208.

13. The polypeptide of claim 11, having the amino acid sequence of part or all of SEQ ID NO:2.

14. An antibody immunologically specific for the polypeptide of claim 11.

15. An isolated nucleic acid molecule having a sequence substantially the same as SEQ ID NO:3.

16. An isolated protein encoded by an *Arabidopsis thaliana* gene, said protein being a member of an SWI2/SNF2 family of polypeptides, loss of function of said protein being associated with DNA hypomethylation.

17. The protein of claim 16, encoded by a gene located on *A. thaliana* chromosome 5, lower arm, centromerically flanked within 20 kilobases by a zinc-finger-encoding gene and telomerically within one kilobase by a gene encoding a glutamic acid tRNA.

18. The protein of claim 16, encoded by a DNA segment on a recombinant cosmid C38, having ATCC Accession No. 207208.

19. The protein of claim 16, having amino acid SEQ ID NO:2.

20. A transgenic organism comprising the nucleic acid molecule of claim 1.

21. The transgenic organism of claim 20, which is a plant.

22. A method of stabilizing fidelity of DNA methylation in an organism, comprising transforming the organism with the nucleic acid molecule of claim 1.

23. A method of reducing or eliminating gene silencing in a plant, comprising inhibiting or preventing expression of an endogenous *DDM1* gene of the plant.

24. A method of introducing inbreeding depression in a plant, comprising inhibiting or preventing expression of an endogenous *DDM1* gene of the plant.

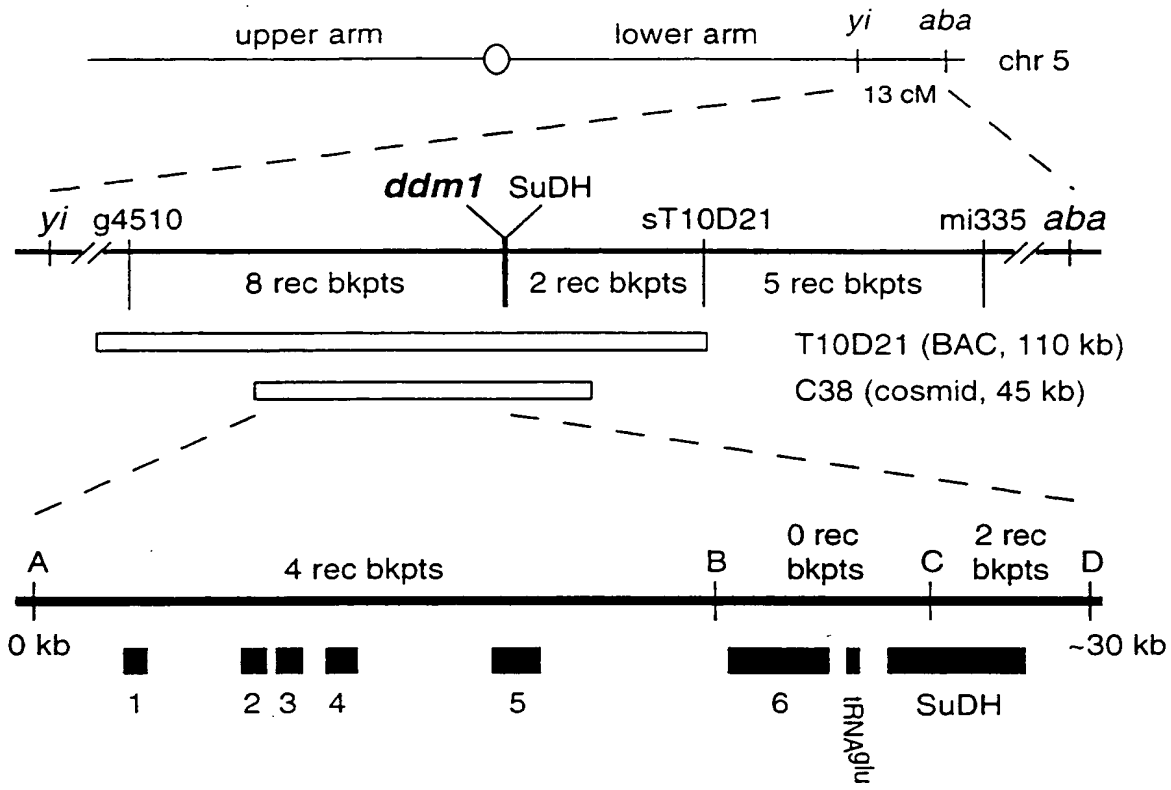
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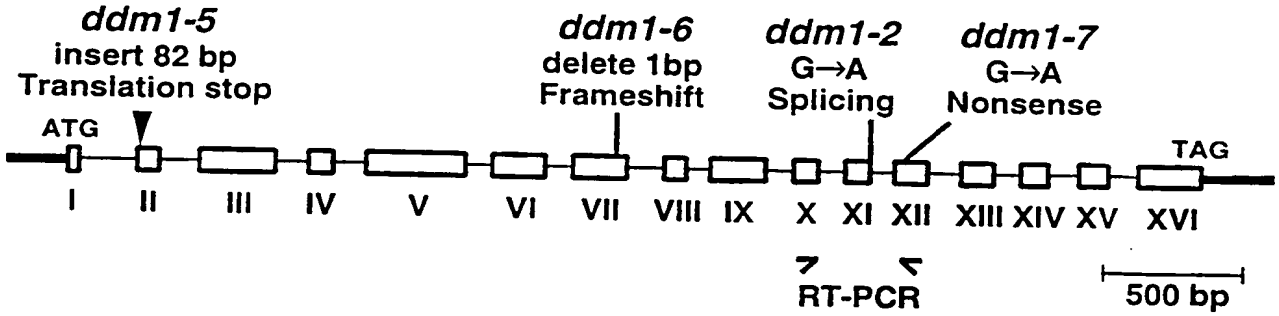
**Figure 1**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RT-PCR
500 bp

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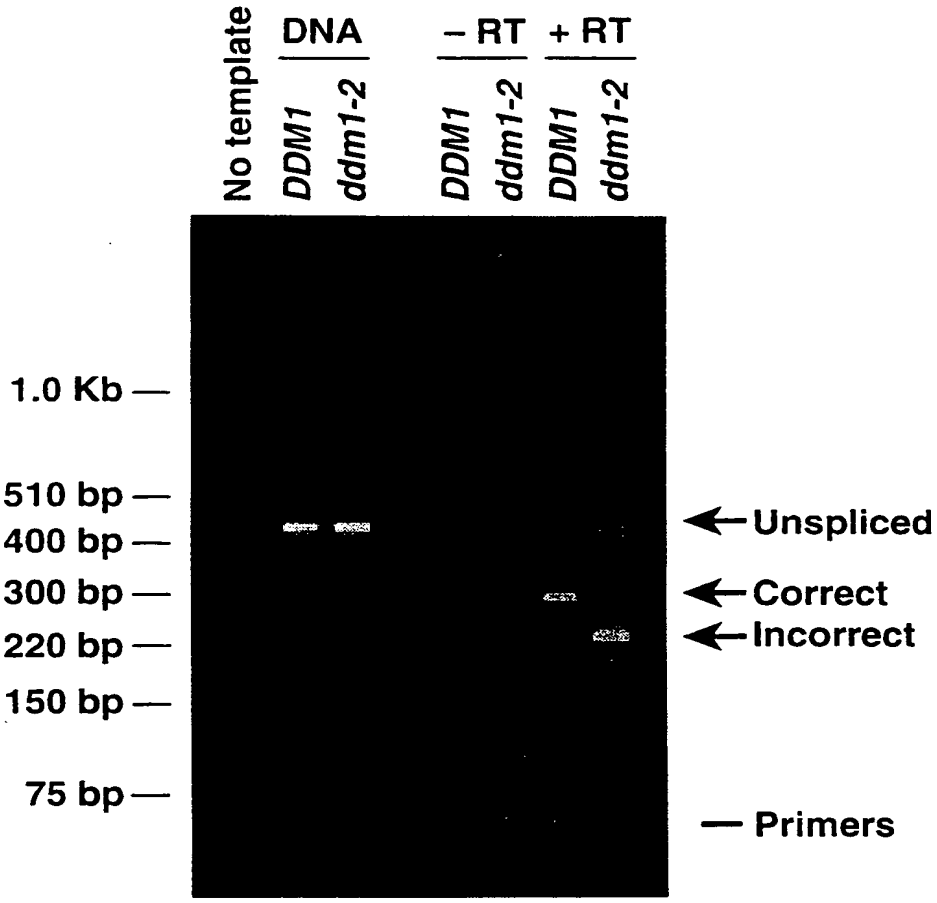
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Fig. 2B

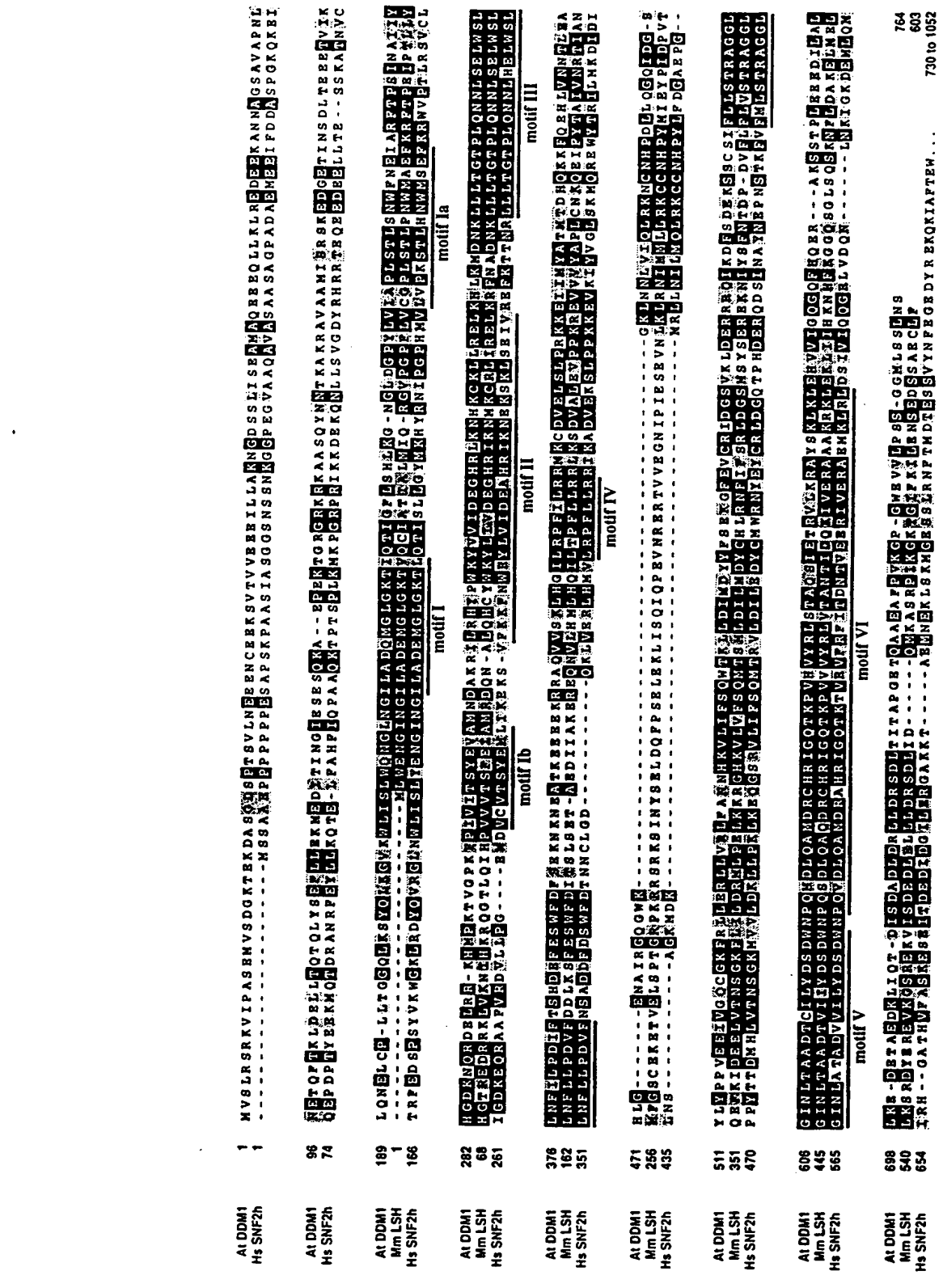


Figure 3

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/09268

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C07K14/415 C07K16/16 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | JEDDELOH, J.A., ET AL. : "the DNA methylation locus DDM1 is required for maintenance of gene silencing in Arabidopsis" GENES AND DEVELOPMENT, vol. 12, no. 11, 1 June 1998 (1998-06-01), pages 1714-1725, XP002114097 the whole document --- | 23,24 |
| X | MITTELSTEN-SCHEID, O., ET AL. : "release of epigenetic gene silencing by trans-acting mutations in Arabidopsis" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 95, January 1998 (1998-01), pages 632-637, XP002114098 cited in the application the whole document --- | 23,24 |

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

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| 2 September 1999 | 15/09/1999 |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | KAKUTANI, T., ET AL. : "developmental abnormalities and epimutations associated with DNA hypomethylation mutations" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 93, October 1996 (1996-10), pages 12406-12411, XP002114099 page 12407, left column; page 12409, left column; Fig. 3 --- | 1-6,10 |
| Y | KAKUTANI, T., ET AL.: "characterization of an Arabidopsis thaliana hypomethylation mutant" NUCLEIC ACID RESEARCH, vol. 23, no. 1, 1995, pages 130-137, XP002049118 cited in the application abstract, last paragraph --- | 1-6,10 |
| A | KAKUTANI, T.: "genetic characterization of late-flowering traits induced by DNA hypomethylation mutation in Arabidopsis thaliana" THE PLANT JOURNAL, vol. 12, no. 6, 1997, pages 1447-1451, XP002114100 abstract, page 1448, right column --- | 1-24 |
| A | ROUNSLEY, S.D., ET AL. : "a BAC end sequence database for identifying minimal overlaps in Arabidopsis genomic sequencing . Update 4." EMBL SEQUENCE DATA LIBRARY, 29 May 1998 (1998-05-29), XP002114101 heidelberg, germany accession no. AQ010627 --- | 1-24 |
| A | VONGS, A., ET AL. : "Arabidopsis thaliana DNA methylation mutants" SCIENCE, vol. 260, June 1993 (1993-06), pages 1926-1928, XP002049119 cited in the application the whole document --- | 1-24 |
| A | WO 98 04725 A (UNIV YALE) 5 February 1998 (1998-02-05) abstract, page 10-14; examples 2 + 3, claims; --- -/-- | 1-24 |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | PASZKOWSKI, J., ET AL.: "plant genes: the genetics of epigenetics" CURRENT BIOLOGY, vol. 8, no. 6, March 1998 (1998-03), pages R206-R208, XP002114102 the whole document | 1-24 |
| P,X | NAKAMURA, Y.: "structural analysis of Arabidopsis thaliana chromosome 5. IX. - unpublished" EMBL SEQUENCE DATA LIBRARY, 7 October 1998 (1998-10-07), XP002114103 heidelberg, germany accession no. AB018119 | 1,2,10, 15 |

# INTERNATIONAL SEARCH REPORT

nformation on patent family members

Inte· ·tional Application No
PC·/US 99/09268

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9804725 A | 05-02-1998 | AU 4048097 A | 20-02-1998 |
| | | EP 0935666 A | 18-08-1999 |

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US99/09268

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| 60/083,612 | 30 April 1998 (30.04.98) | US |
| 09/104,070 | 24 June 1998 (24.06.98) | US |

(71) Applicant *(for all designated States except US)*: WASHINGTON UNIVERSITY [US/US]; 600 South Euclid Avenue, St. Louis, MO 63110 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: RICHARDS, Eric, J. [US/US]; 4446 Westminster Place, St. Louis, MO 63108 (US). JEDDELOH, Jeffrey, A. [US/US]; 6756 W. Lakeridge Drive, New Market, MD 21774 (US).

(74) Agents: REED, Janet, E. et al.; Dann, Dorfman, Herrell and Skillman, Suite 720, 1601 Market Street, Philadelphia, PA 19103 (US).

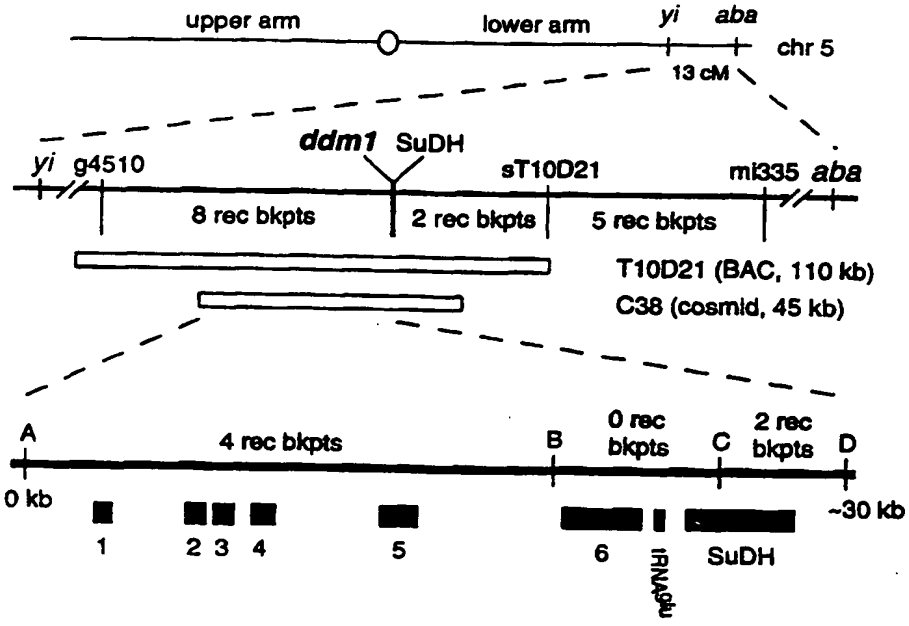
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published**

*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: PLANT GENE THAT REGULATES DNA METHYLATION




(57) Abstract

A novel gene, *DDM1*, and its encoded protein are provided. The gene was isolated from a region of *Arabidopsis thaliana* chromosome 5. *DDM1* appears to be part of the SWI2/SNF2 family of chromatin–remodeling proteins. Disruption of the gene results in DNA hypomethylation, among other phenotypes. The *DDM1* gene defines a novel member of the DNA methylation system. Methods of using *DDM1*, and transgenic organisms comprising *DDM1*, are also provided.

*(Referred to in PCT Gazette No. 8/2000, Section II)

BNSDOCID: <WO___9955891A1_IA>

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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: RICHARDS, Eric, J. [US/US]; 4446 Westminster Place, St. Louis, MO 63108 (US). JEDDELOH, Jeffrey, A. [US/US]; 6756 W. Lakeridge Drive, New Market, MD 21774 (US).

(74) Agents: REED, Janet, E. et al.; Dann, Dorfman, Herrell and Skillman, Suite 720, 1601 Market Street, Philadelphia, PA 19103 (US).

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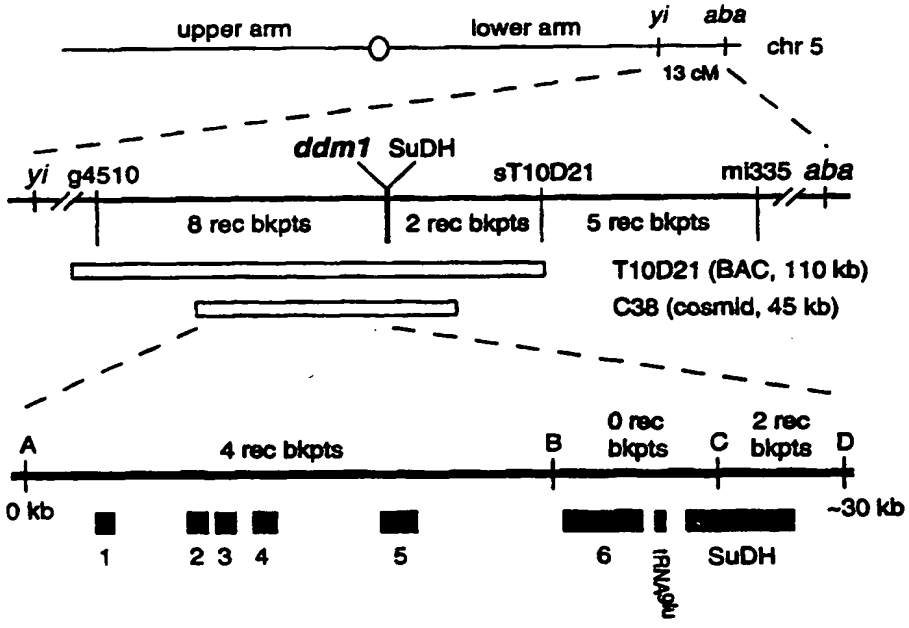


(57) Abstract

A novel gene, *DDM1*, and its encoded protein are provided. The gene was isolated from a region of *Arabidopsis thaliana* chromosome 5. *DDM1* appears to be part of the SWI2/SNF2 family of chromatin–remodeling proteins. Disruption of the gene results in DNA hypomethylation, among other phenotypes. The *DDM1* gene defines a novel member of the DNA methylation system. Methods of using *DDM1*, and transgenic organisms comprising *DDM1*, are also provided.

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# PLANT GENE THAT REGULATES DNA METHYLATION

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the National Science Foundation, Grant Nos. MCB9306266 and BIR9256779.

This application claims priority to U.S. Provisional Application Serial No. 60/_____, filed April 30, 1998, and to U.S. Application No. 09/104,070, filed June 24, 1998 the entireties of which are incorporated by reference herein.

## FIELD OF THE INVENTION

This invention relates to the field of plant molecular biology, genetic engineering and regulation of gene expression. In particular, this invention provides a novel gene, *DDM1*, which plays an important role in the regulation of DNA methylation, and resultant regulation of gene expression, in plant genomic DNA.

## BACKGROUND OF THE INVENTION

Various publications or patents are cited in this application to describe the state of the art to which the invention pertains. Each of these publications or patents is incorporated by reference herein.

Plant genomes contain substantial amounts of 5-methylcytosine. Up to 20-30% of the cytosines are methylated in the nuclear genome of many flowering plants. As in other organisms, methylation of cytosine

residues in plants occurs post-replicatively through the action of cytosine-DNA methyltransferases. Plant DNA methyltransferases have been characterized biochemically, and plant genes encoding these enzymes have been isolated by virtue of their similarity to their mammalian counterparts.

Investigations of native plant genes and transgenic plants containing foreign genes have found a general correlation between transcriptional inactivity and increased DNA methylation, consistent with evidence from mammalian systems. This evidence supports a role for cytosine methylation in maintaining transcriptional states.

The plant's need for developmental plasticity and environmental interaction suggests that plants extensively employ epigenetic regulatory strategies. Such strategies rely on heritable, often reversible, changes in access to the underlying genetic information, but not alteration of the primary nucleotide sequence. As one example, the alteration of DNA methylation is expected to perturb plant development significantly, provided that differential DNA methylation is an important component of epigenetic regulation in plants.

One paradigm linking DNA methylation and developmental regulation comes from work on the mouse, where average genome cytosine methylation levels in embryonic lineages drop sharply in the early cleavages following fertilization, then rise again around the time of implantation. In plants, a similar pattern has been observed in studies of DNA methylation content in pollen and post-embryonic tissue of varying age. Information from such studies indicates that there is a gradual rise in 5-methylcytosine levels in post-embryonic tissues

produced by meristems at positions further from the base of the plant (i.e., tissues of increasing age). Genetic studies of transposon systems in maize also demonstrate an age-dependent gradient of increasing epigenetic modification, which is correlated with DNA methylation.

Both biochemical and genetic approaches have been taken to alter DNA methylation in eucaryotic organisms. Methylation inhibitor treatments have induced developmental abnormalities in many plant species. Transgenic plants expressing antisense molecules specific for a native cytosine methyltransferase gene have been found to exhibit genomic hypomethylation, presumably due to the antisense interference with expression of the gene.

In another approach, mutants of *Arabidopsis thaliana* have been isolated, which show a decrease in DNA methylation (*ddm*) resulting in reduced nuclear 5-methylcytosine levels. The best characterized mutations define the *DDM1* gene. Homozygotes carrying recessive *ddm1* alleles contain 30% of the wild-type levels of 5-methylcytosine. The *ddm1* mutations do not map to the two known cytosine-DNA methyltransferase genes of *A. thaliana*, nor do they affect DNA methyltransferase activity detectable in nuclear extracts (Kakutani et al., *Nuc. Acids Res.* **23**: 130-137, 1995). In addition, *ddm1* mutations do not appear to affect the metabolism of the active methyl group donor, S-adenosylmethionine (Kakutani et al., 1995, *supra*).

For the foregoing reasons, the *DDM1* gene product is likely to be a novel component of the DNA methylation system, or involved in determining the cellular context (e.g., chromatin structure, subnuclear localization) of the methylation reaction. Consequently,

it would be a clear advance in the art of plant molecular and cellular biology to identify and isolate the *DDM1* gene and/or its encoded protein. Such a gene and protein would find utility for the purpose of modifying the methylation status of a selected genome and thereby altering one or more regulatory features of gene expression from that genome.

**SUMMARY OF THE INVENTION**

A novel gene, *DDM1*, and its encoded protein are provided in accordance with the present invention. The gene has been identified as a novel element of the DNA methylation system.

In one aspect of the invention, an isolated nucleic acid molecule comprising a gene located on *Arabidopsis thaliana* chromosome 5, lower arm, is provided. The gene occupies a segment of chromosome 5, lower arm, which is flanked on the centromeric side within 20 kilobases by a gene encoding a zinc-finger protein and on the telomeric side within 1 kilobase by a gene encoding a glutamic acid tRNA. Disruption of the gene is associated with DNA hypomethylation. The gene encodes a polypeptide of about 764 amino acids in length. The nucleotide sequence of the *DDM1* gene is set forth herein as SEQ ID NO:1 and its deduced amino acid sequence as SEQ ID NO:2. In SEQ ID NO:1, the regions of the gene that comprise coding sequence are indicated.

In another aspect of the invention, an isolated *DDM1* gene is provided, having a sequence selected from the group consisting of: (a) SEQ ID NO:1; (b) an allelic variant or natural mutant of SEQ ID NO:1; (c) a sequence hybridizing with part or all of SEQ ID NO:1 or its complement and encoding a polypeptide substantially the

same as part or all of a polypeptide encoded by SEQ ID NO:1; (d) a sequence encoding part or all of a polypeptide having amino acid SEQ ID NO:2; and (e) a sequence encoding part or all of a polypeptide contained in the cosmid clone C38, designated ATCC Accession No. 207208.

According to another aspect of the invention, a polypeptide is provided, which is produced by expression of an isolated nucleic acid molecule comprising part or all of an open reading frame of a gene located on *Arabidopsis thaliana* chromosome 5, lower arm, the gene occupying a segment of chromosome 5, lower arm, flanked on the centromeric side within 20 kilobases by a gene encoding a zinc-finger protein and on the telomeric side within 1 kilobase by a gene encoding a glutamic acid tRNA. This polypeptide preferably has the amino acid sequence of part or all of SEQ ID NO:2.

According to another aspect of the invention, an isolated protein encoded by an *Arabidopsis thaliana* gene is provided, which is a member of an SWI2/SNF2 family of polypeptides. Loss of function of the protein is associated with DNA hypomethylation. The protein is encoded by a gene located on A. *thaliana* chromosome 5, lower arm, centromerically flanked within 20 kilobases by a zinc finger-encoding gene and telomerically within one kilobase by a gene encoding a glutamic acid tRNA.

According to another aspect of the invention, a transgenic organism comprising the *DDM1* gene is provided. In one embodiment, the transgenic organism is a plant.

In other aspects of the invention, methods are provided for stabilizing fidelity of DNA methylation in an organism, which comprise transforming the organism with the *DDM1* gene. Methods are also provided for

reducing or eliminating gene silencing in a plant, or for inducing inbreeding depression in a plant, which comprise inhibiting or preventing expression of an endogenous *DDM1* gene of the plant.

These aspects of the invention, as well as other features and advantages of the invention, will be described in greater detail in the description and examples set forth below.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1.** Map-based isolation of the *A. thaliana DDM1* gene. A genetic map of the region of *A. thaliana* chromosome 5 containing the *DDM1* gene is shown at the top of the figure (see Example 1). The relative sizes of the genetic intervals were determined by the number of recombination breakpoints (rec bkpts) scored in a panel of recombinant lines containing cross-overs between flanking markers *yi* and *aba*. The regions represented in genomic clones T10D21 and C38 are denoted by the open boxes below the genetic map. The ~30 kb interval containing the *DDM1* gene, defined by the genetic markers A and D, is shown at the bottom of the figure. The number of recombination breakpoints scored between markers A - D and *ddm1-2* are indicated. The position of predicted coding regions in the interval are numbered and shown below the physical map. BAC, bacterial artificial chromosome; SuDH, succinate dehydrogenase structural gene.

**Figure 2.** *DDM1* gene structure and identification. **Fig. 2A:** The intron/exon structure of the *DDM1* gene. Protein-coding exons are shown as open boxes, with the start and stop codons indicated. Introns are depicted as thin lines. The position and nature of

four *ddm1* alleles are indicated above the exon/intron map. **Fig. 2B:** RT-PCR analysis of *ddm1-2* and wild-type *DDM1* transcripts. The approximate positions of oligonucleotide primers used in the analysis are shown below the map in Fig. 2A. Amplifications were done on either genomic templates (DNA), first-strand cDNA templates (+RT, plus reverse transcriptase), or mock-synthesized cDNA (-RT, minus reverse transcriptase). Amplified products were separated on a 3% agarose gel and visualized after ethidium bromide staining. Amplification from cDNA representing the properly spliced transcript resulted in a ~280 bp product. The nucleotide sequence of the ~220 bp product amplified from *ddm1-2* cDNA template indicated that the mutation leads to use of an alternate splice donor 56 bp upstream of the wild-type splice donor site.

**Figure 3.** The *A. thaliana DDM1* gene encodes a SWI2/SNF2-like protein. The deduced primary amino acid sequence of DDM1 (At DDM1) is aligned with two other SWI2/SNF2-like protein sequences, *Mus musculus* lymphocyte specific helicase (Mm LSH; SEQ ID NO:4) and human SNF2h (Hs SNF2h; SEQ ID NO:5). Sequence identities are indicated by black boxes and conservative changes are shaded. The positions of the eight signature motifs characteristic of SNF2 family proteins are indicated below the aligned sequences. Amino acid coordinates are indicated on the left; only the N-terminal 730 amino acids (of 1052 total) are shown for human SNF2h, though SEQ ID NO:5 shows the entire protein sequence. The deletion/frameshift caused by the *ddm1-2* allele occurs at amino acid 524. The *ddm1-6* frameshift occurs at amino acid 379, leading to translation of an additional 52 amino acids out of frame. The *ddm1-7* nonsense mutation

occurs at amino acid 549. Dashes indicate gaps introduced by the CLUSTAL W algorithm to maximize alignment (Thompson et al., Nucleic Acids Res. **22**: 4673-4680, 1994). The alignment was processed by BOXSHADE v. 3.21.

## DETAILED DESCRIPTION OF THE INVENTION

### I. Definitions

Various terms relating to the biological molecules of the present invention are used throughout the specification and claims.

With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule.

With respect to RNA molecules of the invention the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to protein, the term "isolated protein" or "isolated and purified protein" is sometimes

used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight the compound of interest (e.g., nucleic acid, oligonucleotide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-99% by weight, the compound of interest. Purity is measured by methods appropriate for the compound of interest (e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus define the differences. In the comparisons made in the present invention, the CLUSTLW program and parameters employed therein were utilized (Thompson et al., 1994, *supra*). However, equivalent alignments and similarity/identity assessments can be obtained through the use of any standard alignment software. For instance, the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program may also be used to compare sequence identity and similarity.

The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variation that do not materially affect the nature of the

protein (i.e. the structure, stability characteristics, substrate specificity and/or biological activity of the protein). With particular reference to nucleic acid sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

With respect to antibodies, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

With respect to oligonucleotides or other single-stranded nucleic acid molecules, the term "specifically hybridizing" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the regulatory sequences necessary for expression of the coding sequence are placed in a nucleic acid molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement other transcription control elements (e.g. enhancers) in an expression vector.

Transcriptional and translational control sequences are DNA regulatory sequences, such as

promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In particular, as used herein, the term "DNA transcriptional response element" refers to a DNA sequence specifically recognized for binding by a DNA binding protein characterized as a transcriptional regulator (either activator or suppressor).

The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA". Such a

nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

The term "reporter gene" refers to genetic sequences which may be operably linked to a promoter region forming a transgene, such that expression of the reporter gene coding region is regulated by the promoter and expression of the transgene is readily assayed.

The term "selectable marker gene" refers to a gene product that when expressed confers a selectable phenotype, such as antibiotic resistance, on a transformed cell or plant.

The term "DNA construct" is sometimes used herein to refer to genetic sequence used to transform plants and generate progeny transgenic plants. These constructs may be administered to plants in a viral or plasmid vector. Other methods of delivery such as Agrobacterium T-DNA mediated transformation and transformation using the biolistic process are also contemplated to be within the scope of the present invention. The transforming DNA may be prepared according to standard protocols such as those set forth in "Current Protocols in Molecular Biology", eds. Frederick M. Ausubel et al., John Wiley & Sons, 1995.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA construct when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and plant cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells

through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

## II. Description of *DDM1* and its Encoded Polyeptide

In accordance with the present invention, a novel gene, *DDM1*, has been isolated from the flowering plant *Arabidopsis thaliana*. Through analysis of mutant plants, this gene has been identified as important for the maintenance of proper genomic cytosine methylation, and its function appears to be necessary to maintain gene silencing. Biochemical and molecular genetic results indicate that *DDM1* encodes a novel component of the DNA methylation machinery.

We have isolated the *DDM1* gene from *A. thaliana* using a map-based cloning approach, which is described in detail in Example 1 and shown in Figure 1. Briefly, the *DDM1* gene was initially localized to the bottom of the lower arm of chromosome 5 by reference to molecular markers segregating in an F2 family (parental cross: Columbia *ddm1/ddm1* X Landsberg erecta *DDM1/DDM1*). Next, recombination breakpoints in the region surrounding a *ddm1* mutation were isolated by collecting cross-over chromosomes by reference to flanking genetic markers. The recombination breakpoints delimited a region of approximately 30 kilobases. Cloned DNA corresponding to this genomic region was isolated by subcloning DNA from a

bacterial artificial chromosome (BAC) containing molecular markers mapping both proximal and distal to the *ddm1* marker. The nucleotide sequence of a single cosmid subclone encompassing the 30 kb region was determined to identify six candidate genes, in addition to a tRNA gene and a previously identified succinate dehydrogenase structural gene.

The search for the *DDM1* gene focused on predicted genes 5 and 6, which fell in the center of the genetic interval defined by recombination breakpoints with the *ddm1-2* marker. The *DDM1* gene was identified as predicted gene 6 based on DNA sequence alterations in four *ddm1* alleles (Figure 2). The EMS-generated *ddm1-2* mutation is a G to A transition in the splice donor site of intron 11 that forces the use of an alternate splice donor site 56 bp upstream in exon 11 (Fig. 2B). The splicing defect leads to a deletion, a frameshift and premature translation termination upstream of predicted functional domains. The fast neutron-generated *ddm1-5* (previously named *som8*; Mittelsten Scheid, O., Afsar, K. & Paszkowski, J. *Proc. Natl. Acad. Sci. USA* **95**: 632-637, 1998).) allele contains an 82 bp insertion (1 bp deleted and replaced with 83 bp) in the second protein-coding exon, leading to an in-frame stop after 30 codons (15 wild-type codons plus 15 codons from the insertion). Premature translation termination is also predicted to result from two additional fast neutron alleles: *ddm1-6* (*som4*) corresponds to a frameshift (1 bp deletion) in exon 7 and *ddm1-7* (*som5*) is a nonsense mutation in exon 12. All four characterized *ddm1* alleles are expected to destroy or severely reduce gene function.

The wild-type *DDM1* gene encodes a predicted protein of 764 amino acids with a high degree of

similarity to SWI2/SNF2-like proteins. Members of the SWI2/SNF2 family are involved in various functions, including transcriptional co-activation, transcriptional co-repression, chromatin assembly and DNA repair. Underlying these apparently diverse activities is the modification or disruption of protein-DNA interactions by multi-protein complexes which contain SWI2/SNF2-like components. Figure 3 shows an alignment among the deduced amino acid sequences of *A. thaliana* DDM1 and two mammalian members of the SNF2 family, human SNF2h (SEQ ID NO:4; Arihara, T. *et al.*, *Cytogenet. Cell Genet.* **81**, 191-193, 1998) and murine LSH (SEQ ID NO:5; lymphocyte specific helicase, LSH; Jarvis, C.D. *et al.* *Gene* **169**, 203-207, 1996). DDM1 contains the eight sequence motifs diagnostic of SWI2/SNF2 family members (Bork, P. & Koonin, E.V. *Nucleic Acids Res.* **21**, 751-752, 1993). *A. thaliana* DDM1 and human SNF2h share 45 percent identity over the approximately 470 amino acid region comprising the signature motifs. Over a similar region, *A. thaliana* DDM1 and murine LSH display approximately 50 percent identity, omitting the 47 residues (amino acids 276-322) apparently unique to LSH. Initial molecular phylogenetic analysis placed DDM1 in a small subfamily, within the SNF2 family, which contains proteins of unknown function, including murine LSH (Eisen, J.A. *et al.* *Nucleic Acids Res.* **23**, 2715-2723, 1995). The proteins of known function most closely related to DDM1 are involved in chromatin remodeling and are grouped in the SNF2L/ISWI subfamily (Eisen *et al.*, 1995, *supra*).

Without intending to be bound by any particular mechanism for the functionality of the *DDM1* gene product, analysis of the foregoing data indicates that the *DDM1* protein functions in the DNA methylation system by

affecting chromatin structure. Two general models for the *DDM1* action are envisioned. The *DDM1* protein may function as a transcriptional co-activator, similar to many SWI2/SNF2-like proteins, to increase the expression of a component of the DNA methylation system. *DDM1* does not affect DNA methyltransferase expression directly because *ddm1* mutant extracts contain wild-type methyltransferase activity (Kakutani et al., 1995, *supra*). However, an unidentified positive effector of DNA methylation may be a target. Alternatively, wild-type *DDM1* function may change chromatin structure to direct certain sequences to the methylation machinery or to facilitate the methylation of genomic substrates. The recently discovered interplay between cytosine methylation and histone acetylation , and the association of SWI2/SNF2-like proteins and histone deacetylases in chromatin remodeling complexes, makes it plausible that *DDM1* affects DNA methylation through modulation of histone modification or another aspect of chromatin structure. Another possibility is that *DDM1* plays a more direct role as a part of a nucleosome remodeling complex that increases the accessibility of the DNA methyltransferase to the hemimethylated substrates in newly replicated chromatin. The latter model is particularly attractive because it predicts that *ddm1* mutations will preferentially hypomethylate genomic sequences packaged in highly condensed chromatin while causing slower loss of methylation in more accessible sequences, consistent with the observed hypomethylation specificity of *ddm1* mutations. The isolation of the *Arabidopsis DDM1* gene in accordance with the present invention points to the importance of chromatin dynamics in the maintenance of cytosine methylation patterns and

identifies a novel component of the eukaryotic DNA methylation pathway.

A number of applications are contemplated for the novel gene of the invention and its encoded protein, and the discovery of the involvement of a *SWI2/SNF2*-like gene in the eucaryotic DNA methylation system. Such applications are described in greater detail below.

Although the *DDM1* genomic clone from *Arabidopsis thaliana* is described and exemplified herein, this invention is intended to encompass nucleic acid sequences and proteins from other organisms, including plants, yeast, insects and mammals, that are sufficiently similar to be used instead of the *Arabidopsis DDM1* nucleic acid and proteins for the purposes described below. These include, but are not limited to, allelic variants and natural mutants of SEQ ID NO:1, which are likely to be found in different species of plants or varieties of *Arabidopsis*. Because such variants are expected to possess certain differences in nucleotide and amino acid sequence, this invention provides an isolated *DDM1* nucleic acid molecule having at least about 60% (preferably 70% and more preferably over 80%) sequence homology in the coding regions with the nucleotide sequence set forth as SEQ ID NO:1 (and, most preferably, specifically comprising the coding region of SEQ ID NO:1). This invention also provides isolated polypeptide products of the open reading frames of SEQ ID NO:1, having at least about 60% (preferably 70% or 80% or greater) sequence homology with the amino acid sequences of SEQ ID NO:2. Because of the natural sequence variation likely to exist among *DDM1* genes, one skilled in the art would expect to find up to about 30-40% nucleotide sequence variation, while still maintaining

the unique properties of the *DDM1* gene and encoded polypeptide of the present invention. Such an expectation is due in part to the degeneracy of the genetic code, as well as to the known evolutionary success of conservative amino acid sequence variations, which do not appreciably alter the nature of the encoded protein. Accordingly, such variants are considered substantially the same as one another and are included within the scope of the present invention.

The following description sets forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (1999) (hereinafter "Ausubel et al.") are used.

## A. Preparation of *DDM1* Nucleic Acid Molecules, encoded Polypeptides and Antibodies Specific for the Polypeptides

### 1. Nucleic Acid Molecules

*DDM1* nucleic acid molecules of the invention may be prepared by two general methods: (1) they may be synthesized from appropriate nucleotide triphosphates, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art.

The availability of nucleotide sequence information, such as the cDNA having SEQ ID NO:1, enables preparation of an isolated nucleic acid molecule of the

invention by oligonucleotide synthesis. Synthetic oligonucleotides may be prepared by the phosphoramadite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC). Long, double-stranded polynucleotides, such as a DNA molecule of the present invention, must be synthesized in stages, due to the size limitations inherent in current oligonucleotide synthetic methods. Thus, for example, a long double-stranded molecule may be synthesized as several smaller segments of appropriate complementarity. Complementary segments thus produced may be annealed such that each segment possesses appropriate cohesive termini for attachment of an adjacent segment. Adjacent segments may be ligated by annealing cohesive termini in the presence of DNA ligase to construct an entire long double-stranded molecule. A synthetic DNA molecule so constructed may then be cloned and amplified in an appropriate vector.

*DDM1* genes also may be isolated from appropriate biological sources using methods known in the art. In the exemplary embodiment of the invention, the *A. thaliana DDM1* clone was isolated from a BAC genomic library of *A. thaliana* In alternative embodiments, cDNA clones of *DDM1* may be isolated. A preferred means for isolating *DDM1* genes is PCR amplification using genomic templates and *DDM1*-specific primers.

In accordance with the present invention, nucleic acids having the appropriate level sequence homology with part or all the coding regions of SEQ ID NO:1 may be identified by using hybridization and washing conditions of appropriate stringency. For example,

hybridizations may be performed, according to the method of Sambrook et al., using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 2X SSC and 0.1% SDS; (4) 2 hours at 45-55°in 2X SSC and 0.1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989):

$T_m = 81.5°C + 16.6\text{Log}[Na+] + 0.41(\%\ G+C) - 0.63\ (\%\ \text{formamide}) - 600/\#\text{bp in duplex}$

As an illustration of the above formula, using [N+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the $T_m$ is 57°C. The $T_m$ of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C. Such a sequence would be considered substantially homologous to the sequences of the present invention.

Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in plasmid cloning/expression vector, such as pGEM-T (Promega Biotech, Madison, WI) or pBluescript (Stratagene, La Jolla, CA), either of which is propagated in a suitable

*E. coli* host cell.

*DDM1* nucleic acid molecules of the invention include cDNA, genomic DNA, RNA, and fragments thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a nucleic acid molecule of the present invention, such as selected segments of the DNA having SEQ ID NO:1. Such oligonucleotides are useful as probes for detecting *DDM1* genes or mRNA in test samples, e.g. by PCR amplification, or for the positive or negative regulation of expression of *DDM1* genes at or before translation of the mRNA into proteins.

The *DDM1* promoter and other expression regulatory sequences for *DDM1* are also expected to be useful in connection with the present invention. SEQ ID NO:1 shows about 550 bp of sequence upstream from the beginning of the coding region, which should contain such expression regulatory sequences. In addition, SEQ ID NO:3 constitutes about 5 kbp of additional upstream sequence, which should contain other regulatory sequences, such as enhancer elements.

## 2. Proteins

Polypeptides encoded by *DDM1* nucleic acids of the invention may be prepared in a variety of ways, according to known methods. If produced *in situ* the polypeptides may be purified from appropriate sources, e.g., plant parts.

Alternatively, the availability of nucleic acid molecules encoding the polypeptides enables production of the proteins using *in vitro* expression methods known in the art. For example, a cDNA or gene may be cloned into

an appropriate *in vitro* transcription vector, such a pSP64 or pSP65 for *in vitro* transcription, followed by cell-free translation in a suitable cell-free translation system, such as wheat germ or rabbit reticulocytes. *In vitro* transcription and translation systems are commercially available, e.g., from Promega Biotech, Madison, Wisconsin or BRL, Rockville, Maryland.

According to a preferred embodiment, larger quantities of *DDM1*-encoded polypeptide may be produced by expression in a suitable procaryotic or eucaryotic system. For example, part or all of a DNA molecule, such as the coding portion of SEQ ID NO:1, may be inserted into a plasmid vector adapted for expression in a bacterial cell (such as *E. coli*) or a yeast cell (such as *Saccharomyces cerevisiae*), or into a baculovirus vector for expression in an insect cell. Such vectors comprise the regulatory elements necessary for expression of the DNA in the host cell, positioned in such a manner as to permit expression of the DNA in the host cell. Such regulatory elements required for expression include promoter sequences, transcription initiation sequences and, optionally, enhancer sequences.

The DDM1 polypeptide produced by gene expression in a recombinant procaryotic or eucyarotic system may be purified according to methods known in the art. In a preferred embodiment, a commercially available expression/secretion system can be used, whereby the recombinant protein is expressed and thereafter secreted from the host cell, to be easily purified from the surrounding medium. If expression/secretion vectors are not used, an alternative approach involves purifying the recombinant protein by affinity separation, such as by immunological interaction with antibodies that bind specifically to the recombinant protein. Such methods are commonly used by skilled practitioners.

The *DDM1*-encoded polypeptides of the invention, prepared by the aforementioned methods, may be analyzed according to standard procedures. Methods for analyzing the functional activity are available. For instance, DNA methylation levels are detectable by known methods. Alternatively, the function of the *DDM1* gene product as part of a chromatin remodeling machine permits the use of *in vitro* assays for chromatin remodeling, which are known in the art (e.g., B.R. Cairns, *Trends in Biochem.* **23**: 20-25, 1998).

The present invention also provides antibodies capable of immunospecifically binding to polypeptides of the invention. Polyclonal or monoclonal antibodies directed toward the polypeptide encoded by *DDM1* may be prepared according to standard methods. Monoclonal antibodies may be prepared according to general methods of Köhler and Milstein, following standard protocols. In a preferred embodiment, antibodies are prepared, which react immunospecifically with various epitopes of the *DDM1*-encoded polypeptides.

## B. Uses of *DDM1* Nucleic Acids, Encoded Proteins and Antibodies

### 1. *DDM1* Nucleic Acids

*DDM1* nucleic acids may be used for a variety of purposes in accordance with the present invention. The DNA, RNA, or fragments thereof may be used as probes to detect the presence of and/or expression of *DDM1* genes. Methods in which *DDM1* nucleic acids may be utilized as probes for such assays include, but are not limited to: (1) *in situ* hybridization; (2) Southern hybridization (3) northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR).

The *DDM1* nucleic acids of the invention may also be utilized as probes to identify related genes from

other species, including but not limited to, plants, yeast, insects and mammals, including humans. As is well known in the art and described above, hybridization stringencies may be adjusted to allow hybridization of nucleic acid probes with complementary sequences of varying degrees of homology. Thus, *DDM1* nucleic acids may be used to advantage to identify and characterize other genes of varying degrees of relation to the exemplary coding sequence of SEQ ID NO:1, thereby enabling further characterization of this family of genes. Additionally, they may be used to identify genes encoding proteins that interact with protein encoded by *DDM1* (e.g., by the "interaction trap" technique).

As discussed above and in greater detail in Example 1, the similarity among plant *DDM1* and its *SWI2/SNF2* counterparts in yeast, *Drosophila* and mammals indicates that the functional aspects of these proteins will also be conserved. Thus, *DDM1* is expected to play an important role in DNA methylation and resultant down-regulation of gene expression. Plants engineered to over-express *DDM1* can be expected to have improved fidelity of the DNA methylation system. The evidence suggests that loss of DDM1 function leads to reduction in the efficiency of maintenance methylation due to reduced accessibility of the methyltransferase enzyme to the substrate. Hence, excess DDM1 function could lead to an increase in the fidelity of the inheritance of DNA methylation thereby reducing the occurrence of spurious methylation mistakes which could compromise the organism's viability or fecundity. In fact, there are experimental data demonstrating that loss of DDM1 function leads to stochastic hypermethylation, and epigenetic lesion formation, as well. For these reasons, *DDM1* overexpression lines are expected to have useful properties.

Transgenic plants expressing the *DDM1* gene or antisense nucleotides can be generated using standard plant transformation methods known to those skilled in the art. These include, but are not limited to, *Agrobacterium* vectors, PEG treatment of protoplasts, biolistic DNA delivery, UV laser microbeam, gemini virus vectors, calcium phosphate treatment of protoplasts, electroporation of isolated protoplasts, agitation of cell suspensions with microbeads coated with the transforming DNA, direct DNA uptake, liposome-mediated DNA uptake, and the like. Such methods have been published in the art. See, e.g., Methods for Plant Molecular Biology (Weissbach & Weissbach, eds., 1988); Methods in Plant Molecular Biology (Schuler & Zielinski, eds., 1989); Plant Molecular Biology Manual (Gelvin, Schilperoort, Verma, eds., 1993); and Methods in Plant Molecular Biology - A Laboratory Manual (Maliga, Klessig, Cashmore, Gruissem & Varner, eds., 1994).

The method of transformation depends upon the plant to be transformed. The biolistic DNA delivery method is useful for nuclear transformation. In another embodiment of the invention, *Agrobacterium* vectors are used to advantage for efficient transformation of plant nuclei.

In a preferred embodiment, the gene is introduced into plant nuclei in *Agrobacterium* binary vectors. Such vectors include, but are not limited to, BIN19 (Bevan, 1984) and derivatives thereof, the pBI vector series (Jefferson et al., 1987), and binary vectors pGA482 and pGA492 (An, 1986).

The *DDM1* gene may be placed under a powerful constitutive promoter, such as the Cauliflower Mosaic Virus (CaMV) 35S promoter or the figwort mosaic virus 35S promoter. Transgenic plants expressing the *DDM1* gene under an inducible promoter (either its own promoter or a

heterologous promoter) are also contemplated to be within the scope of the present invention. Inducible plant promoters include the tetracycline repressor/operator controlled promoter.

Using an *Agrobacterium* binary vector system for transformation, the *DDM1* coding region, under control of a constitutive or inducible promoter as described above, is linked to a nuclear drug resistance marker, such as kanamycin resistance. *Agrobacterium*-mediated transformation of plant nuclei is accomplished according to the following procedure:

(1) the gene is inserted into the selected *Agrobacterium* binary vector;

(2) transformation is accomplished by co-cultivation of plant tissue (e.g., leaf discs) with a suspension of recombinant *Agrobacterium*, followed by incubation (e.g., two days) on growth medium in the absence of the drug used as the selective medium (see, e.g., Horsch et al. 1985);

(3) plant tissue is then transferred onto the selective medium to identify transformed tissue; and

(4) identified transformants are regenerated to intact plants.

It should be recognized that the amount of expression, as well as the tissue specificity of expression of the *DDM1* gene in transformed plants can vary depending on the position of their insertion into the nuclear genome. Such position effects are well known in the art. For this reason, several nuclear transformants should be regenerated and tested for expression of the transgene.

In some instances, it may be desirable to down-regulate or inhibit expression of endogenous *DDM1* in plants possessing the gene. One clear benefit to engineering a reduction of DDM1 function is to reduce

gene (including transgene) silencing. Plant lines with reduced or absent DDM1 function are expected to be viable based on results obtained with *Arabidopsis*. Further, it has been shown that gene silencing is suppressed in *ddm1* *Arabidopsis* lines (Jeddeloh et al., *Genes Devel.* **12**:1714-1725, 1998). There are two other beneficial characteristics of *DDM1* deficient plant lines. First, alteration in DNA methylation leads to changes in flowering time, and as such, is a potentially powerful tool for manipulating plant development. (See, e.g., Richards, *Trends in Genetics* **13**: 319-323, 1998), Second, *ddm1* mutant lines exhibit inbreeding depression (a reduction in vigor after inbreeding) (Richards, Trends in Genetics, 1998, *supra*), a characteristic which may be desirable to include in situations where proprietary germplasms in hybrid plants are at risk of unauthorized use. For instance, a genetically engineered hybrid (containing one or more useful transgenes) could be further engineered to down-regulate endogenous *DDM1* expression. Unauthorized inbreeding of such lines would be discouraged because the progeny of such lines would lack vigor.

To achieve the aforementioned benefits associated with reduced gene expression, *DDM1* nucleic acid molecules, or fragments thereof, may also be utilized to control the production of *DDM1*-encoded proteins. In one embodiment, full-length *DDM1* antisense molecules or antisense oligonucleotides, targeted to specific regions of *DDM1*-encoded RNA that are critical for translation, are used. The use of antisense molecules to decrease expression levels of a pre-determined gene is known in the art. In a preferred embodiment, antisense molecules are provided *in situ* by transforming plant cells with a DNA construct which, upon

transcription, produces the antisense sequences. Such constructs can be designed to produce full-length or partial antisense sequences.

In another embodiment, overexpression of *DDM1* is induced to generate a co-suppression effect. This excess expression serves to promote down-regulation of both endogenous and exogenous *DDM1* genes.

Optionally, transgenic plants can be created containing mutations in the region encoding the active site of *DDM1*. This embodiment may be preferred in certain instances.

From the foregoing discussion, it can be seen that *DDM1* and its homologs will be useful for introducing alterations in gene expression in an organism, for a variety of purposes. As described above, for instance, the *Arabidopsis DDM1* gene can be used to isolate mutants or engineer organisms that express reduced function of *DDM1* orthologs. Based on results in *Arabidopsis*, such mutants or engineered organisms are expected to be viable and display valuable characteristics, such as inbreeding depression and a reduction in gene silencing. In addition, we anticipate that dysfunction in human *DDM1* orthologs may contribute to diseases that involve alterations in DNA methylation, including cancer (Baylin, S.B. et al., *Adv. Cancer Res.* **72**: 141-196, 1998) and immunodeficiency/ chromosome instability/facial anomalies syndrome (ICF) (Smeets, D.F.C.M. et al., *Hum. Genet.* **94**: 240-246, 1994).

## 2. DDM1 Proteins and Antibodies

Purified *DDM1*-encoded proteins, or fragments thereof, may be used to produce polyclonal or monoclonal antibodies which also may serve as sensitive detection reagents for the presence and accumulation of*DDM1*-encoded

protein in cultured cells or tissues and in intact organisms. Recombinant techniques enable expression of fusion proteins containing part or all of the *DDM1*-encoded protein. The full length protein or fragments of the protein may be used to advantage to generate an array of monoclonal or polyclonal antibodies specific for various epitopes of the protein, thereby providing even greater sensitivity for detection of the protein in cells or tissue.

DDM1 gene products also may be useful as pharmaceutical agents if it is determined that DDM1 loss of function plays a role in carcinogenesis, as mentioned above. The gene products could be administered as replacement therapy for persons having neoplasias associated with DDM1 loss of function.

Polyclonal or monoclonal antibodies immunologically specific for *DDM1*-encoded proteins may be used in a variety of assays designed to detect and quantitate the protein. Such assays include, but are not limited to: (1) flow cytometric analysis; (2) immunochemical localization in cultured cells or tissues; and (3) immunoblot analysis (e.g., dot blot, Western blot) of extracts from various cells and tissues.

Polyclonal or monoclonal antibodies that immunospecifically interact with the polypeptide encoded by *DDM1* can be utilized for identifying and purifying such proteins. For example, antibodies may be utilized for affinity separation of proteins with which they immunospecifically interact. Antibodies may also be used to immunoprecipitate proteins from a sample containing a mixture of proteins and other biological molecules.

The following specific examples are provided to illustrate embodiments of the invention. They are not intended to limit the scope of the invention in any way.

## EXAMPLE 1
## Map-Based Isolation of the *Arabidopsis thaliana DDM1* Gene

**Construction of recombination breakpoint lines.** The recombination breakpoint lines were assembled in the F3 generation from a parental cross between *YI DDM1 ABA/YI ddm1-2 ABA* (Columbia strain (Col)) and *yi DDM1 aba/yi DDM1 aba* (Landsberg erecta strain (La er)). The recessive *yi* mutation leads to a yellow inflorescence. The recessive *aba* mutation causes a defect in abscisic acid biosynthesis and a wilting phenotype. Information on genetic markers and the *A. thaliana* genetic map can be found at: http://genome-www.stanford.edu/Arabidopsis/. Selfed seeds from F1 *YI ddm1-2 ABA/yi DDM1 aba* plants were collected and 135 F2 recombinants (yi ABA, yellow inflorescence, non-wilting; or YI aba: green inflorescence, wilting) were identified. Selfed seeds from 111 of the 135 recombinant F2 individuals were planted to generate F3 tissue for genomic DNA preparation. The genotype at the *DDM1* locus was scored in the F3 generation by Southern blot analysis using methylation-sensitive endonucleases as described previously (Vongs, A., Kakutani, T., Martienssen, R.A. & Richards, E.J. , *Science* **260**: 1926-1928, 1993).

**Molecular markers.** Two of the molecular markers shown in Figure 1 were available from the *Arabidopsis* research community: g4510 (*Arabidopsis* Biological Resource Center (ABRC) stock# CD2-38) and mi335 (ABRC stock# CD3-288). The remainder of the molecular markers shown in Figure 1 were developed in accordance with the present invention. sT10D21Bam is an

insert end subclone of the BAC (bacterial artificial chromosome) clone T10D21 constructed by complete cleavage with *Bam*HI and recircularization. sT10D21Bam recognizes a Col/La er *Pst*I RFLP (restriction fragment length polymorphism). Molecular marker A is an *Xba*I Col/La er RFLP marker recognized by a 5.7 kb *Hin*dIII fragment of the C38 cosmid insert. Marker B is a *Rsa*I Col/La er CAPS marker (Koneiczny & Ausubel, Plant J. **4**: 403-410, 1993) (forward primer: 5'-TCAAGGAGATGATTCGGGCGT-3', SEQ ID NO: 6; reverse primer: 5'-AAAGGACCCATTTACAGAACAC-3', SEQ ID NO:7). The remaining markers, C and D, correspond to RFLP's (*Bcl*I and *Pst*I, respectively) recognized by the succinate dehydrogenase cDNA clone, 105N23T7 (ABRC stock# 105N23T7).

**Genomic library construction and screening.** We screened the available *A. thaliana* BAC genomic libraries by standard colony hybridization techniques using radiolabeled 105N23T7 insert as a probe. The clone we subsequently focused upon, T10D21, came from the Texas A&M University BAC library (Choi et al., *Weeds World* **2**: 17-20, 1995). To facilitate subsequent analysis, we cloned *Sau*3AI partially digested fragments from the T10D21 insert into the *Bam*HI site of SuperCos (Stratagene). We chose to further characterize one member of the resulting cosmid sublibrary, C38, which contained genetic markers that flanked *ddm1-2*. The C38 cosmid was submitted on April 20, 1999, under the provisions of the Budapest Treaty, with the American Type Culture Collection (Manassas VA), and assigned ATCC Accession No. 207208.

## EXAMPLE 2
## *DDM1* Gene Structure and Identification; Sequence Determination of *DDM1* Gene

**DNA sequence determination.** C38 cosmid (~45 kb) DNA, prepared using Qiagen columns and protocols, was sonicated and 1-2 kb fragments isolated from a low-melting temperature agarose gel. The size-selected DNA was cloned into the *Sma*I site of a M13mp18 vector to generate a shotgun library suitable for DNA sequence determination. Single-stranded substrates were prepared and sequenced using conventional dye-terminator cycle sequencing protocols (Perkin-Elmer) on either an ABI 373 or ABI 377 automated DNA sequencer. The DNA sequence of the *ddm1* alleles was determined using PCR-amplified templates and oligonucleotide primers dispersed throughout the *DDM1* gene. Sequence assembly and analysis were accomplished using Phred/Phrap/Consed (http://www.mbt.washington.edu/) and DNASTAR software suites.

**RT-PCR cDNA analysis.** *DDM1* gene structure was determined by analysis of the genomic DNA sequence and the nucleotide sequence of RT-PCR (reverse transcription-polymerase chain reaction) products encompassing the coding region. *DDM1* and *ddm1-2* transcripts were analyzed by RT-PCR as follows. Total RNA was prepared using the Qiagen RNeasy™ protocol. Poly(A)+ transcripts were collected on oligo-$d(T)_{25}$ magnetic Dynabeads (Dynal) and first-strand cDNA synthesis performed following Dynal protocols using Stratascript (Stratagene) reverse transcriptase. Aliquots of the bead-immobilized first-strand cDNA library were used as templates for PCR amplification using KlenTaqI polymerase (Clontech). The following oligonucleotide primers were used for the RT-PCR experiment shown in Fig. 2*b*: forward, 5'-GCTGGAAGGGAAAGCTTAACAACC-3' (SEQ ID NO:8); reverse,

5'-ACACTGCCATCGATTCTGCAAACC-3' (SEQ ID NO:9).

**GenBank accession numbers and SEQ ID NOS.**

*Arabidopsis DDM1* genomic DNA sequence: SEQ ID NO:1;

*Arabidopsis* DDM1 deduced amino acid sequence: SEQ ID NO:2;

*Arabidopsis DDM1* 5' upstream genomic DNA sequence: SEQ ID NO:3;

*Mus musculus* lymphocyte specific helicase (LSH); Genbank Accession No. AAB08015; SEQ ID NO:4;

*Homo sapiens* SNF2h; Genbank Accession No. AB010882; SEQ ID NO:5;

succinate dehydrogenase cDNA 105N23T7, T22529;

primers: SEQ ID NOS: 6-9.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

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Jeffrey A. Jeddeloh

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Glu Ala Met Ala Gln Glu Glu Glu Gln Leu Leu Lys Leu Arg Glu Asp
65                  70                  75                  80
Glu Glu Lys Ala Asn Asn Ala Gly Ser Ala Val Ala Pro Asn Leu Asn
                85                  90                  95
Glu Thr Gln Phe Thr Lys Leu Asp Glu Leu Leu Thr Gln Thr Gln Leu
            100                 105                 110
Tyr Ser Glu Phe Leu Leu Glu Lys Met Glu Asp Ile Thr Ile Asn Gly
        115                 120                 125
Ile Glu Ser Glu Ser Gln Lys Ala Glu Pro Glu Lys Thr Gly Arg Gly
    130                 135                 140
Arg Lys Arg Lys Ala Ala Ser Gln Tyr Asn Asn Thr Lys Ala Lys Arg
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Ala Val Ala Ala Met Ile Ser Arg Ser Lys Glu Asp Gly Glu Thr Ile
                165                 170                 175
Asn Ser Asp Leu Thr Glu Glu Glu Thr Val Ile Lys Leu Gln Asn Glu
            180                 185                 190
Leu Cys Pro Leu Leu Thr Gly Gly Gln Leu Lys Ser Tyr Gln Leu Lys
        195                 200                 205
Gly Val Lys Trp Leu Ile Ser Leu Trp Gln Asn Gly Leu Asn Gly Ile
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Leu Ala Asp Gln Met Gly Leu Gly Lys Thr Ile Gln Thr Ile Gly Phe
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Ala Pro Leu Ser Thr Leu Ser Asn Trp Phe Asn Glu Ile Ala Arg Phe
            260                 265                 270
Thr Pro Ser Ile Asn Ala Ile Ile Tyr His Gly Asp Lys Asn Gln Arg
        275                 280                 285
Asp Glu Leu Arg Arg Lys His Met Pro Lys Thr Val Gly Pro Lys Phe
    290                 295                 300
Pro Ile Val Ile Thr Ser Tyr Glu Val Ala Met Asn Asp Ala Lys Arg
305                 310                 315                 320
Ile Leu Arg His Tyr Pro Trp Lys Tyr Val Val Ile Asp Glu Gly His
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Arg Leu Lys Asn His Lys Cys Lys Leu Leu Arg Glu Leu Lys His Leu
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Lys Met Asp Asn Lys Leu Leu Leu Thr Gly Thr Pro Leu Gln Asn Asn
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Ile Gly Gln Gly Gln Phe His Gln Glu Arg Ala Lys Ser Ser Thr Pro
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Leu Glu Glu Glu Asp Ile Leu Ala Leu Leu Lys Glu Asp Glu Thr Ala
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    50                  55                  60
Leu Leu Tyr His Gly Thr Arg Glu Asp Arg Arg Lys Leu Val Lys Asn
65                  70                  75                  80
Ile His Lys Arg Gln Gly Thr Leu Gln Ile His Pro Val Val Val Thr
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Ser Phe Glu Ile Ala Met Arg Asp Gln Asn Ala Leu Gln His Cys Tyr
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Trp Lys Tyr Leu Ile Val Asp Glu Gly His Arg Ile Lys Asn Met Lys
        115                 120                 125
Cys Arg Leu Ile Arg Glu Leu Lys Arg Phe Asn Ala Asp Asn Lys Leu
    130                 135                 140
Leu Leu Thr Gly Thr Pro Leu Gln Asn Asn Leu Ser Glu Leu Trp Ser
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Leu Leu Asn Phe Leu Leu Pro Asp Val Phe Asp Asp Leu Lys Ser Phe
                165                 170                 175
Glu Ser Trp Phe Asp Ile Thr Ser Leu Ser Glu Thr Ala Glu Asp Ile
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225                 230                 235                 240
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Phe Gly Ser Cys Glu Lys Glu Thr Val Glu Leu Ser Pro Thr Gly Arg
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Pro Lys Arg Arg Ser Arg Lys Ser Ile Asn Tyr Ser Glu Leu Asp Gln
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Phe Pro Ser Glu Leu Glu Lys Leu Ile Ser Gln Ile Gln Pro Glu Val
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Asn Arg Glu Arg Thr Val Val Glu Gly Asn Ile Pro Ile Glu Ser Glu
305                 310                 315                 320
Val Asn Leu Lys Leu Arg Asn Ile Met Met Leu Leu Arg Lys Cys Cys
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Asn His Pro Tyr Met Ile Glu Tyr Pro Ile Asp Pro Val Thr Gln Glu
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Val Phe Ser Gln Met Thr Ser Met Leu Asp Ile Leu Met Asp Tyr Cys
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His Leu Arg Asn Phe Ile Phe Ser Arg Leu Asp Gly Ser Met Ser Tyr
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Ser Glu Arg Glu Lys Asn Ile Tyr Ser Phe Asn Thr Asp Pro Asp Val
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Phe Leu Phe Leu Val Ser Thr Arg Ala Gly Gly Leu Gly Ile Asn Leu
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Thr Ala Ala Asp Thr Val Ile Ile Tyr Asp Ser Asp Trp Asn Pro Gln
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<400> 5

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Ala Ser Ala Gly Pro Ala Asp Ala Glu Met Glu Glu Ile Phe Asp Asp
    50                  55                  60
Ala Ser Pro Gly Lys Gln Lys Glu Ile Gln Glu Pro Asp Pro Thr Tyr
65                  70                  75                  80
Glu Glu Lys Met Gln Thr Asp Arg Ala Asn Arg Phe Glu Tyr Leu Leu
                85                  90                  95
Lys Gln Thr Glu Leu Phe Ala His Phe Ile Gln Pro Ala Ala Gln Lys
            100                 105                 110
Thr Pro Thr Ser Pro Leu Lys Met Lys Pro Gly Arg Pro Arg Ile Lys
        115                 120                 125
Lys Asp Glu Lys Gln Asn Leu Leu Ser Val Gly Asp Tyr Arg His Arg
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Arg Thr Glu Gln Glu Glu Asp Glu Glu Leu Leu Thr Glu Ser Ser Lys
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Tyr Arg Asn Ile Pro Gly Pro His Met Val Leu Val Pro Lys Ser Thr
225                 230                 235                 240
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Ser Val Cys Leu Ile Gly Asp Lys Glu Gln Arg Ala Ala Phe Val Arg
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Asp Val Leu Leu Pro Gly Glu Trp Asp Val Cys Val Thr Ser Tyr Glu
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Glu Lys Ser Leu Pro Pro Lys Lys Glu Val Lys Ile Tyr Val Gly Leu
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        515                 520                 525
Leu Asp Gly Gln Thr Pro His Asp Glu Arg Gln Asp Ser Ile Asn Ala
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Tyr Asn Glu Pro Asn Ser Thr Lys Phe Val Phe Met Leu Ser Thr Arg
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Arg Val Ser Glu Pro Lys Ala Pro Lys Ala Pro Arg Pro Pro Lys Gln
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Pro Asn Val Gln Asp Phe Gln Phe Phe Pro Pro Arg Leu Phe Glu Leu
    770                 775                 780
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785                 790                 795                 800
Pro Arg Asn Pro Glu Leu Pro Asn Ala Ala Gln Ala Gln Lys Glu Glu
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Gln Leu Lys Ile Asp Glu Ala Glu Ser Leu Asn Asp Glu Glu Leu Glu
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Glu Lys Glu Lys Leu Leu Thr Gln Gly Phe Thr Asn Trp Asn Lys Arg
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    850                 855                 860
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865                 870                 875                 880
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                885                 890                 895
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Arg Arg Ile Ser Ile Lys Lys Ala Leu Asp Thr Lys Ile Gly Arg Tyr
        915                 920                 925
Lys Ala Pro Phe His Gln Leu Arg Ile Ser Tyr Gly Thr Asn Lys Gly
    930                 935                 940
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Lys Leu Gly Phe Asp Lys Glu Asn Val Tyr Asp Glu Leu Arg Gln Cys
                965                 970                 975
Ile Arg Asn Ser Pro Gln Phe Arg Phe Asp Trp Phe Leu Lys Ser Arg
            980                 985                 990
Thr Ala Met Glu Leu Gln Arg Arg Cys Asn Thr Leu Ile Thr Leu Ile
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acactgccat cgattctgca aacc 24

**We claim:**

1. An isolated nucleic acid molecule comprising a gene located on *Arabidopsis thaliana* chromosome 5, lower arm, said gene occupying a segment of said chromosome 5, lower arm, flanked on the centromeric side within 20 kilobases by a gene encoding a zinc-finger protein and on the telomeric side within 1 kilobase by a gene encoding a glutamic acid tRNA, the disruption of said gene being associated with DNA hypomethylation.

2. The nucleic acid molecule of claim 1, wherein said gene is composed of exons that form an open reading frame having a sequence that encodes a polypeptide about 750-850 amino acids in length.

3. A cDNA molecule comprising the exons of the nucleic acid molecule of claim 2.

4. The nucleic acid molecule of claim 2, wherein said open reading frame encodes an amino acid sequence substantially the same as SEQ ID NO:2.

5. The nucleic acid molecule of claim 4, wherein said open reading frame encodes amino acid SEQ ID NO:2.

6. The nucleic acid molecule of claim 5, which comprises an open reading frame of SEQ ID NO:1.

7. A recombinant DNA molecule, comprising a vector having an insert that includes the nucleic acid molecule of claim 1.

8. The recombinant DNA molecule of claim 7,

which is cosmid C38, ATCC Accession No. 207208.

9. An oligonucleotide between about 10 and 100 nucleotides in length, which specifically hybridizes with a portion of the nucleic acid molecule of claim 1.

10. An isolated nucleic acid molecule which is a gene, the disruption of which is associated with DNA hypomethylation, having a sequence selected from the group consisting of:

a) SEQ ID NO:1;

b) an allelic variant or natural mutant of SEQ ID NO:1;

c) a sequence hybridizing with part or all of SEQ ID NO:1 or its complement and encoding a polypeptide substantially the same as part or all of a polypeptide encoded by SEQ ID NO:1;

d) a sequence encoding part or all of a polypeptide having amino acid SEQ ID NO:2; and

e) a sequence encoding part or all of a polypeptide contained in the cosmid clone C38, designated ATCC Accession No. 207208.

11. A polypeptide produced by expression of an isolated nucleic acid molecule comprising part or all of an open reading frame of a gene located on *Arabidopsis thaliana* chromosome 5, lower arm, said gene occupying a segment of said chromosome 5, lower arm, flanked on the centromeric side within 20 kilobases by a gene encoding a zinc-finger protein and on the telomeric side within 1 kilobase by a gene encoding a glutamic acid tRNA, the disruption of said gene being associated with DNA hypomethylation.

12. The polypeptide of claim 11, produced by

expression of a sequence selected from the group consisting of:

a) SEQ ID NO:1;

b) an allelic variant or natural mutant of SEQ ID NO:1;

c) a sequence hybridizing with part or all of SEQ ID NO:1 or its complement and encoding a polypeptide substantially the same as part or all of a polypeptide encoded by SEQ ID NO:1;

d) a sequence encoding part or all of a polypeptide having amino acid SEQ ID NO:2; and

e) a sequence encoding part or all of a polypeptide contained in the clone designated ATCC Accession No. 207208.

13. The polypeptide of claim 11, having the amino acid sequence of part or all of SEQ ID NO:2.

14. An antibody immunologically specific for the polypeptide of claim 11.

15. An isolated nucleic acid molecule having a sequence substantially the same as SEQ ID NO:3.

16. An isolated protein encoded by an *Arabidopsis thaliana* gene, said protein being a member of an SWI2/SNF2 family of polypeptides, loss of function of said protein being associated with DNA hypomethylation.

17. The protein of claim 16, encoded by a gene located on *A. thaliana* chromosome 5, lower arm, centromerically flanked within 20 kilobases by a zinc-finger-encoding gene and telomerically within one kilobase by a gene encoding a glutamic acid tRNA.

18. The protein of claim 16, encoded by a DNA segment on a recombinant cosmid C38, having ATCC Accession No. 207208.

19. The protein of claim 16, having amino acid SEQ ID NO:2.

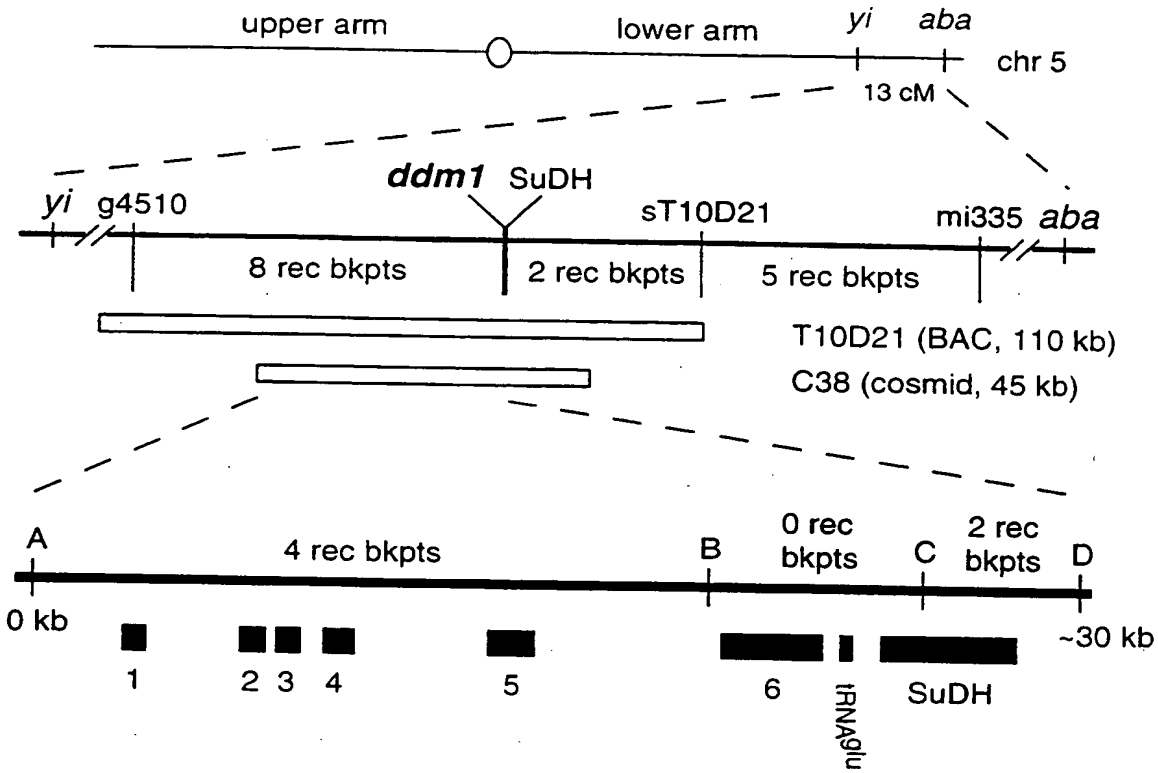
20. A transgenic organism comprising the nucleic acid molecule of claim 1.

21. The transgenic organism of claim 20, which is a plant.

22. A method of stabilizing fidelity of DNA methylation in an organism, comprising transforming the organism with the nucleic acid molecule of claim 1.

23. A method of reducing or eliminating gene silencing in a plant, comprising inhibiting or preventing expression of an endogenous *DDM1* gene of the plant.

24. A method of introducing inbreeding depression in a plant, comprising inhibiting or preventing expression of an endogenous *DDM1* gene of the plant.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C38 (cosmid, 45 kb)
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4 rec bkpts
B
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C
2 rec bkpts
D
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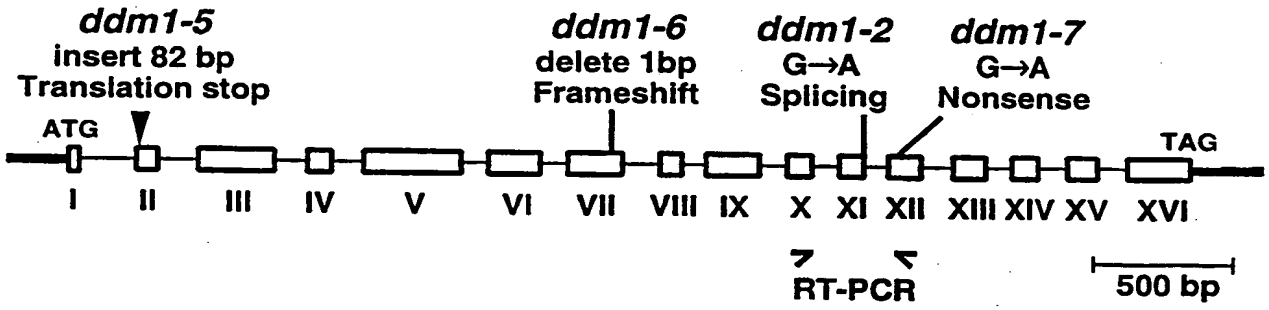
~30 kb
1
2 3 4
5
6
tRNAglu
SuDH

**Figure 1**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Translation stop
ddm1-6
delete 1bp
Frameshift
ddm1-2
G→A
Splicing
ddm1-7
G→A
Nonsense
ATG
TAG
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RT-PCR
500 bp

Fig. 2A

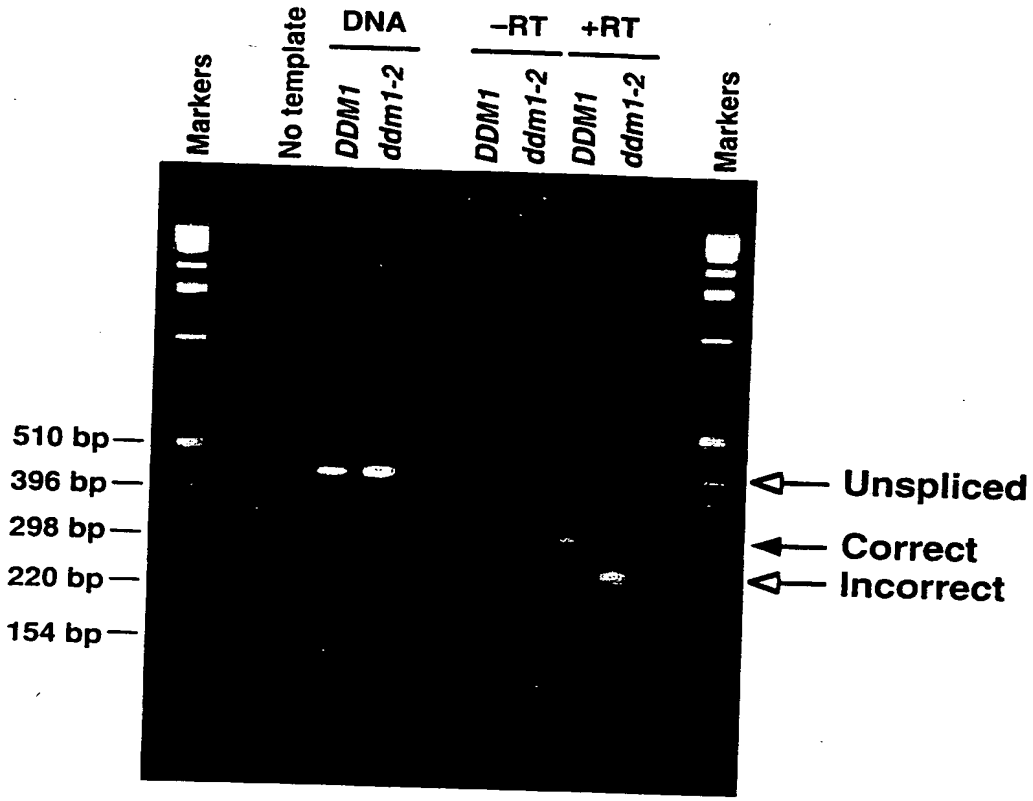


Fig. 2B

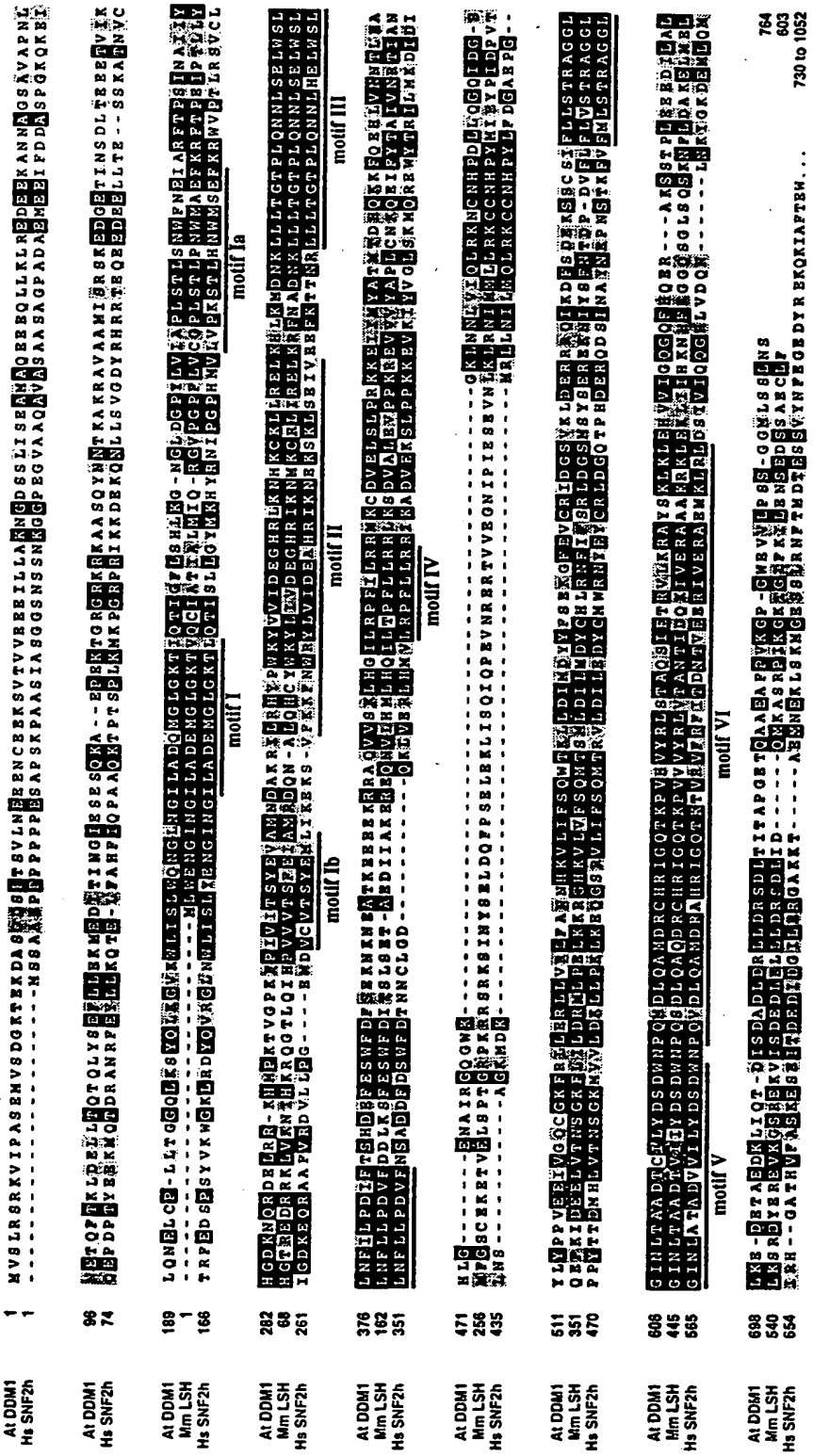


Figure 3

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/09268

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C07K14/415 C07K16/16 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | JEDDELOH, J.A., ET AL. : "the DNA methylation locus DDM1 is required for maintenance of gene silencing in Arabidopsis" GENES AND DEVELOPMENT, vol. 12, no. 11, 1 June 1998 (1998-06-01), pages 1714-1725, XP002114097 the whole document --- | 23,24 |
| X | MITTELSTEN-SCHEID, O., ET AL. : "release of epigenetic gene silencing by trans-acting mutations in Arabidopsis" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 95, January 1998 (1998-01), pages 632-637, XP002114098 cited in the application the whole document --- | 23,24 |
| | -/-- | |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 2 September 1999 | 15/09/1999 |
| Name and mailing address of the ISA<br>European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Authorized officer<br>Holtorf, S |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | KAKUTANI, T., ET AL. : "developmental abnormalities and epimutations associated with DNA hypomethylation mutations" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 93, October 1996 (1996-10), pages 12406-12411, XP002114099 page 12407, left column; page 12409, left column; Fig. 3 --- | 1-6,10 |
| Y | KAKUTANI, T., ET AL.: "characterization of an Arabidopsis thaliana hypomethylation mutant" NUCLEIC ACID RESEARCH, vol. 23, no. 1, 1995, pages 130-137, XP002049118 cited in the application abstract, last paragraph --- | 1-6,10 |
| A | KAKUTANI, T.: "genetic characterization of late-flowering traits induced by DNA hypomethylation mutation in Arabidopsis thaliana" THE PLANT JOURNAL, vol. 12, no. 6, 1997, pages 1447-1451, XP002114100 abstract, page 1448, right column --- | 1-24 |
| A | ROUNSLEY, S.D., ET AL. : "a BAC end sequence database for identifying minimal overlaps in Arabidopsis genomic sequencing . Update 4." EMBL SEQUENCE DATA LIBRARY, 29 May 1998 (1998-05-29), XP002114101 heidelberg, germany accession no. AQ010627 --- | 1-24 |
| A | VONGS, A., ET AL. : "Arabidopsis thaliana DNA methylation mutants" SCIENCE, vol. 260, June 1993 (1993-06), pages 1926-1928, XP002049119 cited in the application the whole document --- | 1-24 |
| A | WO 98 04725 A (UNIV YALE) 5 February 1998 (1998-02-05) abstract, page 10-14; examples 2 + 3, claims; --- | 1-24 |

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# INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/09268

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | PASZKOWSKI, J., ET AL.: "plant genes: the genetics of epigenetics" CURRENT BIOLOGY, vol. 8, no. 6, March 1998 (1998-03), pages R206-R208, XP002114102 the whole document --- | 1-24 |
| P,X | NAKAMURA, Y.: "structural analysis of Arabidopsis thaliana chromosome 5. IX. - unpublished" EMBL SEQUENCE DATA LIBRARY, 7 October 1998 (1998-10-07), XP002114103 heidelberg, germany accession no. AB018119 ----- | 1,2,10, 15 |

# INTERNATIONAL SEARCH REPORT

nformation on patent family members

Inter ntional Application No
PC /US 99/09268

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9804725 A | 05-02-1998 | AU 4048097 A | 20-02-1998 |
| | | EP 0935666 A | 18-08-1999 |

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